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<b>(21) International Application Number:</b> PCT/US98/04703 <b>(22) International Filing Date:</b> 10 March 1998 (10.03.98)  <b>(30) Priority Data:</b> 60/040,376 10 March 1997 (10.03.97) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 60/040,376 (CIP) Filed on 10 March 1997 (10.03.97)  <b>(71) Applicants (for all designated States except US):</b> OTTAWA CIVIC LOEB RESEARCH INSTITUTE [CA/CA]; 725 Parkdale Avenue, Ottawa, Ontario K1Y 4E9 (CA). QIAGEN GMBH [DE/DE]; Max-Vomer-Strasse 4, D-4010 Hilden (DE). THE UNIVERSITY OF IOWA RESEARCH FOUNDATION [US/US]; 214 Technology Innovation Center, Oakdale Research Campus, Iowa City, IA 52242 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DAVIS, Heather, L. [CA/CA]; Loeb Research Institute, Ottawa Civic Hospital, 1053 Carling Avenue, Ottawa, Ontario K1Y 4E9 (CA). SCHORR, Joachim [DE/DE]; Qiagen GmbH,	<b>Max-Vomer-Strasse 3, D-4010 Hilden (DE). KRIEG, Arthur, M. [US/US]; 890 Park Place, Iowa City, IA 52246 (US).</b>  <b>(74) Agent:</b> HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>With amended claims.</i>	
<b>(54) Title:</b> USE OF NUCLEIC ACIDS CONTAINING UNMETHYLATED CpG DINUCLEOTIDE AS AN ADJUVANT		
<b>(57) Abstract</b> <p>The present invention is based on the finding that nucleic acids containing at least one unmethylated cytosine-guanine (CpG) dinucleotide affect immune responses in a subject. These nucleic acids containing at least one unmethylated cytosine-guanine (CpG) dinucleotide can be used to induce an immune response in a subject. The method includes administering to the subject a therapeutically effective amount of nucleic acid encoding an antigenic polypeptide, and a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide. The invention also provides a method for treating a subject having or at risk of having viral-mediated disorder, comprising administering to the subject a therapeutically effective amount of a nucleic acid encoding an antigenic polypeptide and an effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.</p>		

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**USE OF NUCLEIC ACIDS CONTAINING UNMETHYLATED CpG  
DINUCLEOTIDE AS AN ADJUVANT**

**FIELD OF THE INVENTION**

5           This invention relates to generally to adjuvants, and specifically to the use of oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) as an adjuvant.

**BACKGROUND OF THE INVENTION**

10           Bacterial DNA, but not vertebrate DNA, has direct immunostimulatory effects on peripheral blood mononuclear cells (PBMC) *in vitro* (Krieg *et al.*, 1995). This lymphocyte activation is due to unmethylated CpG dinucleotides, which are present at the expected frequency in bacterial DNA (1/16), but are under-represented (CpG suppression, 1/50 to 1/60) and methylated in vertebrate DNA. Activation may also be triggered by  
15           addition of synthetic oligodeoxynucleotides (ODN) that contain an unmethylated CpG dinucleotide in a particular sequence context. It appears likely that the rapid immune activation in response to CpG DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules.

20           CpG DNA induces proliferation of almost all (>95%) B cells and increases immunoglobulin (Ig) secretion. This B cell activation by CpG DNA is T cell independent and antigen non-specific. However, B cell activation by low concentrations of CpG DNA has strong synergy with signals delivered through the B cell antigen receptor for both B  
25           cell proliferation and Ig secretion (Krieg *et al.*, 1995). This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by CpG DNA promotes antigen specific immune responses. In addition to its direct effects on B cells,

CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete a variety of cytokines, including high levels of IL-12 (Klinman *et al.*, 1996; Halpern *et al.*, 1996; Cowdery *et al.*, 1996). These cytokines stimulate natural killer (NK) cells to secrete g-interferon (IFN-g) and have increased lytic activity (Klinman *et al.*, 1996, *supra*;  
5 Cowdery *et al.*, 1996, *supra*; Yamamoto *et al.*, 1992; Ballas *et al.*, 1996). Overall, CpG DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN-g with little secretion of Th2 cytokines (Klinman *et al.*, 1996). The strong direct effects (T cell independent) of CpG DNA on B cells, as well as the induction of cytokines which could have indirect effects on B-cells via T-help pathways, suggests utility of CpG DNA  
10 in the form of ODN as a vaccine adjuvant.

A DNA vaccine induces immune responses against an antigenic protein expressed *in vivo* from an introduced gene. The DNA vaccine is most often in the form of a plasmid DNA expression vector produced in bacteria and then purified and delivered to  
15 muscle or skin (see Vogel and Sarver, 1995; Brazolot Millan and Davis, 1997; Donnelly *et al.*, 1996). DNA vaccines have been demonstrated to show efficacy against numerous viral, bacterial and parasitic diseases in animal models. Almost all studies show induction of very strong and long-lasting humoral and cell-mediated immune responses, and protection against live pathogen challenge (where it could be evaluated). The efficacy of DNA  
20 vaccines is attributed, at least in part, to the continuous *in vivo* synthesis of antigen that leads to efficient antigen presentation. In particular, endogenously-synthesized antigen is presented by class I MHC, leading to induction of CD8+ cytotoxic T lymphocytes (CTL). In contrast, most whole killed and subunit vaccines, where antigen is processed solely in the exogenous form, often fail to induce CTL. More recently however, it has been shown that  
25 the presence of unmethylated CpG motifs in the DNA vaccines is essential for the induction of immune responses against the antigen (Sato *et al.*, 1996).

Hepatitis B virus (HBV) poses a serious world-wide health problem. The current HBV vaccines are subunit vaccines containing particles of HBV envelope protein(s) which

include several B and T cell epitopes known collectively as HBV surface antigen (HBsAg). The HBsAg particles may be purified from the plasma of chronically infected individuals or more commonly are produced as recombinant proteins. These vaccines induce antibodies against HBsAg (anti-HBs), which confer protection if present in titers 10 milli-International Units per milliliter (mIU/ml) (Ellis, 1993). While the subunit vaccines are safe and generally efficacious, they fail to meet all current vaccination needs. For example, early vaccination of infants born to chronically infected mothers, as well as others in endemic areas, drastically reduces the rate of infection, but a significant proportion of these babies will still become chronically infected themselves. This could possibly be reduced if high titers of anti-HBs antibodies could be induced earlier and if there were HBV-specific CTL. In addition, there are certain individuals who fail to respond (non-responders) or do not attain protective levels of immunity (hypo-responders). Finally, there is an urgent need for an effective treatment for the estimated 350 million chronic carriers of HBV and a therapeutic vaccine could meet this need.

#### SUMMARY OF THE INVENTION

The present invention is based on the finding that nucleic acids containing at least one unmethylated cytosine-guanine (CpG) dinucleotide affect the immune response in a subject by activating natural killer cells (NK) or redirecting a subject's immune response from a Th2 to a Th1 response by inducing monocytic and other cells to produce Th1 cytokines. These nucleic acids containing at least one unmethylated CpG can be used as an adjuvant, specifically to induce an immune response against an antigenic protein.

In one embodiment, the invention provides a method of inducing an immune response in a subject by administering to the subject a therapeutically effective amount of a nucleic acid encoding an antigenic protein and a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.

In another embodiment, the invention provides a method for treating a subject having or at risk of having a virally mediated disorder by administering to the subject a

therapeutically effective amount of a nucleic acid encoding an antigenic protein and an effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.

5 In further embodiment, the invention provides a method for treating a subject having or at risk of having a chronic viral infection by administering to the subject an effective amount of an antigenic polypeptide and an effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.

10 In another embodiment, a pharmaceutical composition containing an immunostimulatory CpG oligonucleotide and a nucleic acid encoding an antigenic protein in a pharmaceutically acceptable carrier is provided.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 FIG. 1 is a graph illustrating humoral responses in BALB/c mice immunized with 1 g recombinant HBsAg protein alone, adsorbed onto alum (25 mg  $Al^{3+}$ /mg HBsAg), with 100 g of immunostimulatory CpG ODN, or with both alum and CpG ODN. Each point represents the group mean ( $n=10$ ) for titers of anti-HBs (total IgG) as determined in triplicate by end-point dilution ELISA assay. End-point titers were defined as the highest  
20 plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of control non-immune plasma with a cut-off value of 0.05. The upper graph shows results on a linear scale and the lower graph shows results on a logarithmic scale ( $\log_{10}$ ).

FIG. 2 is a graph illustrating humoral responses in BALB/c mice immunized with  
25 1 g recombinant HBsAg protein with alum and with 0, 10, 100 or 500 g of CpG ODN added. Each point represents the group mean ( $n=10$ ) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 3 is a graph of humoral responses in C57BL/6 mice immunized with 1 g recombinant HBsAg protein without adjuvant, with alum, with 100 g of CpG ODN, or with both alum and CpG ODN. Mice were boosted in the identical fashion after 6 weeks. Each point represents the group mean (n=5) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 4 is a graph of humoral responses in C57BL/6 mice immunized with 1 g recombinant HBsAg protein without adjuvant, or with 1, 10 or 100 g of CpG ODN. Mice were boosted in the identical fashion after 6 weeks. Each point represents the group mean (n=5) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 5 is a graph of humoral responses in B10.S hypo-responder mice immunized with 1 g recombinant HBsAg protein without adjuvant, with alum, and/or with 10 g of CpG ODN. Each point represents the group mean (n=5) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 6 is a graph of humoral responses in C2D non-responder mice immunized with 1 g recombinant HBsAg protein with alum or with alum plus 10 g of CpG ODN. Each point represents the group mean (n=5) for anti-HBs titers (total IgM or IgG) as determined by end-point dilution ELISA assay.

FIG. 7 is a bar graph illustrating humoral responses in C57BL/6 mice at 8 weeks after immunization with 1 g recombinant HBsAg protein without adjuvant, with alum, with 100 g of CpG ODN, or with both alum and CpG ODN. Mice had been boosted in the identical fashion at 6 weeks. Each point represents the group mean (n=5) for anti-HBs titers (IgG1 and IgG2a isotypes) as determined by end-point dilution ELISA assay.

FIG. 8 is a graph of humoral responses in BALB/c mice immunized with 10 g HBsAg-expressing DNA vaccine (pCMV-S) injected alone or with 100 or 500 g of CpG

ODN added. Each point represents the group mean (n=10) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 9 is a graph of humoral responses in B10.S mice immunized with 2 g recombinant HBsAg protein without adjuvant or with 50 g pCMV-S DNA vaccine. Each point represents the group mean (n=5) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 10 is a graph of humoral responses in newborn BALB/c mice immunized with 1 g recombinant HBsAg protein with alum or with 10 g pCMV-S DNA vaccine on the day of birth or 7 days later. Each point represents the group mean (n=10) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 11 is a graph of humoral responses in BALB/c mice primed with 10 g HBsAg-expressing DNA vaccine (pCMV-S) and given 2 g recombinant HBsAg protein at the same time in the same or a different muscle or given the HBsAg 2 or 8 weeks later. Each point represents the group mean (n=10) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is to be understood that this invention is not limited to the particular methodology, protocols, sequences, models and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the oligonucleotides and methodologies which are described in the publications which might be used in connection with the presently described invention.



The binding of DNA to cells has been shown to be similar to a ligand receptor interaction: binding is saturable, competitive, and leads to DNA endocytosis and degradation into oligonucleotides (Bennet, R.M., *et al.*, *J. Clin. Invest.* 76:2182, 1985). Like DNA, oligodeoxyribonucleotides are able to enter cells in a process which is sequence, temperature, and energy independent (Jaroszewski and Cohen, *Ad. Drug Del. Rev.* 6:235, 1991). An "oligodeoxyribonucleotide" as used herein is a deoxyribonucleic acid sequence from about 3-50 bases in length. Lymphocyte oligodeoxyribonucleotide uptake has been shown to be regulated by cell activation (Krieg, A.M., *et al.*, *Antisense Research and Development* 1:161, 1991). The present invention is based on the finding that certain oligonucleotides (ODN) containing at least one unmethylated cytosine-guanine (CpG) dinucleotide activate the immune response.

In one embodiment, the invention provides a method for stimulating an immune response in a subject by administering a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated CpG. The term "nucleic acid" or "oligonucleotide" refers to a polymeric form of nucleotides at least five bases in length. The nucleotides of the invention can be deoxyribonucleotides, ribonucleotides, or modified forms of either nucleotide. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased activity.

The nucleic acid molecule can include the use of phosphorothioate or phosphorodithioate rather than phosphodiesterase linkages within the backbone of the molecule, or methylphosphorothioate terminal linkages (Krieg, A.M., *et al.*, *Antisense and Nucl Acid Drug Dev* 6:133-9, 1996; Boggs, R.T., *et al.*, *Antisense and Nucl Acid Drug Dev*, 7:461-71, 1997). The phosphate backbone modification can occur at the 5' end of the nucleic acid, for example at the first two nucleotides of the 5' end of the nucleic acid. The phosphate backbone modification may occur at the 3' end of the nucleic acid, for example at the last five nucleotides of the 3' end of the nucleic acid. International Patent Application WO 95/26204, entitled "Immune stimulation by phosphorothioate oligonucleotide analogs" reports the nonsequence-specific immunostimulatory effect of phosphorothioate modified

oligonucleotides. Nontraditional bases such as inosine and queosine, as well as acetyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine can also be included, which are not as easily recognized by endogenous endonucleases. Other stabilized nucleic acid molecules include: nonionic DNA analogs, such as alkyl- and aryl-phosphonates (in which the charged oxygen moiety is alkylated). Nucleic acid molecules which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini are also included. The term "oligonucleotide" includes both single and double-stranded forms of DNA.

A "CpG" or "CpG motif" refers to a nucleic acid having a cytosine followed by a guanine linked by a phosphate bond. The term "methylated CpG" refers to the methylation of the cytosine on the pyrimidine ring, usually occurring the 5-position of the pyrimidine ring. The term "unmethylated CpG" refers to the absence of methylation of the cytosine on the pyrimidine ring. Methylation, partial removal, or removal of an unmethylated CpG motif in an oligonucleotide of the invention is believed to reduce its effect. Methylation or removal of all unmethylated CpG motifs in an oligonucleotide substantially reduces its effect. The effect of methylation or removal of a CpG motif is "substantial" if the effect is similar to that of an oligonucleotide that does not contain a CpG motif.

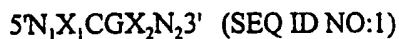
Preferably the CpG oligonucleotide is in the range of about 8 to 30 bases in size. For use in the instant invention, the nucleic acids can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, S.L., and Caruthers, M.H., *Tet. Let.* 22:1859, 1981); nucleoside H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4051-4054, 1986; Froehler *et al.*, *Nucl. Acid. Res.* 14:5399-5407, 1986, ; Garegg *et al.*, *Tet. Let.* 27:4055-4058, 1986, Gaffney *et al.*, *Tet. Let.* 29:2619-2622, 1988). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor laboratory Press, New York, 1989) which after being administered to a subject are degraded into oligonucleotides.

Oligonucleotides can be prepared from existing nucleic acid sequences (*e.g.*, genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

5 For use *in vivo*, nucleic acids are preferably relatively resistant to degradation (*e.g.*, via endo-and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using  
10 automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made, *e.g.*, as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other  
15 DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A., *Chem. Rev.* 90:544, 1990; Goodchild, J., *Bioconjugate Chem.* 1:165, 1990).

For administration *in vivo*, nucleic acids may be associated with a molecule that results in higher affinity binding to target cell (*e.g.*, B-cell, monocytic cell and natural killer  
20 (NK) cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex." Nucleic acids can be ionically or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or cross-linking agents can be used, *e.g.*, protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in  
25 liposomes or virosomes using well-known techniques.

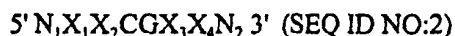
In one embodiment, the nucleic acid sequences useful in the methods of the invention are represented by the formula:



wherein at least one nucleotide separates consecutive CpGs;  $X_1$  is adenine, guanine, or thymidine;  $X_2$  is cytosine or thymine, N is any nucleotide and  $N_1 + N_2$  is from about 0-26 bases. In a preferred embodiment,  $N_1$  and  $N_2$  do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

5 However, nucleic acids of any size (even may kb long) can be used in the invention if CpGs are present, as larger nucleic acids are degraded into oligonucleotides inside cells. Preferred synthetic oligonucleotides do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' or 3' terminals and/or the consensus mitogenic CpG motif is not a  
10 palindrome. A "palindromic sequence" or "palindrome" means an inverted repeat (*i.e.*, a sequence such as ABCDEE'D'C'B'A', in which A and A' are bases capable of forming the usual Watson-Crick base pairs.

In another embodiment, the method of the invention includes the use of an oligonucleotide which contains a CpG motif represented by the formula:



wherein at least one nucleotide separates consecutive CpGs;  $X_1 X_2$  is selected from the group consisting of GpT, GpG, GpA, ApT and ApA;  $X_3 X_4$  is selected from the group consisting of TpT or CpT; N is any nucleotide and  $N_1 + N_2$  is from about 0-26 bases. In a preferred  
20 embodiment,  $N_1$  and  $N_2$  do not contain a CCGG quadmer or more than one CCG or CGG trimer. CpG ODN are also preferably in the range of 8 to 30 bases in length, but may be of any size (even many kb long) if sufficient motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides of this formula do not include a CCGG quadmer or more than one CCG or CGG trimer at or  
25 near the 5' and/or 3' terminals and/or the consensus mitogenic CpG motif is not a palindrome. Other CpG oligonucleotides can be assayed for efficacy using methods described herein. An exemplary nucleic acid sequence of the invention is  
5'-TCCATGACGTTCTGACGTT-3' (SEQ ID NO:3).

A prolonged effect can be obtained using stabilized oligonucleotides, where the  
30 oligonucleotide incorporates a phosphate backbone modification (*e.g.*, a phosphorothioate or

phosphorodithioate modification). More particularly, the phosphate backbone modification occurs at the 5' end of the nucleic acid for example, at the first two nucleotides of the 5' end of the nucleic acid. Further, the phosphate backbone modification may occur at the 3' end of the nucleic acid for example, at the last five nucleotides of the 3' end of the nucleic acid.

5 Preferred nucleic acids containing an unmethylated CpG have a relatively high stimulation with regard to B cell, monocyte, and/or natural killer cell responses (e.g., induction of cytokines, proliferative responses, lytic responses, among others).

*Nucleic acids containing an unmethylated CpG can be effective in any mammal, preferably a human. Different nucleic acids containing an unmethylated CpG can cause optimal immune stimulation depending on the mammalian species. Thus an oligonucleotide causing optimal stimulation in humans may not cause optimal stimulation in a mouse. One of skill in the art can identify the optimal oligonucleotides useful for a particular mammalian species of interest.*

10

The "stimulation index" is a measure of a CpG ODN to effect an immune response which can be tested in various immune cell assays. The stimulation of the immune response can be assayed by measuring various immune parameters, e.g., measuring the antibody-forming capacity, number of lymphocyte subpopulations, mixed leukocyte response assay, lymphocyte proliferation assay. The stimulation of the immune response can also be measured in an assay to determine resistance to infection or tumor growth. Methods for measuring a stimulation index are well known to one of skill in the art. For example, one assay is the incorporation of  $^3\text{H}$  uridine in a murine B cell culture, which has been contacted with a  $20\mu\text{M}$  of oligonucleotide for 20h at  $37^\circ\text{C}$  and has been pulsed with  $1\mu\text{Ci}$  of  $^3\text{H}$  uridine; and harvested and counted 4h later. The induction of secretion of a particular cytokine can also be used to assess the stimulation index. Without meaning to be bound by theory, for use *in vivo*, for example to treat a subject at risk of exposure to a hepatitis virus, it is important that the CpG ODN be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity. In one method, the stimulation index of the CpG ODN with regard to B-cell proliferation is at least about 5,

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preferably at least about 10, more preferably at least about 15 and most preferably at least about 20, while recognizing that there are differences in the stimulation index among individuals.

5           The CpG ODN of the invention stimulate cytokine production (*e.g.*, IL-6, IL-12, IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF). Exemplary sequences include:

TCCATGTCGCTCCTGATGCT (SEQ ID NO:4),  
TCCATGTCGTTTCCTGATGCT (SEQ ID NO:5), and  
TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:6).

10          The CpG ODN of the invention are also useful for stimulating natural killer cell (NK) lytic activity in a subject such as a human. Specific, but nonlimiting examples of such sequences include:

TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:7),  
TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:6),  
15       TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:8),  
GCGTGCGTTGTCGTTGTCGTT (SEQ ID NO:9),  
TGTCGTTTGTCGTTTGTCGTT (SEQ ID NO:10),  
TGTCGTTGTCGTTGTCGTT (SEQ ID NO:11), and  
TCGTCGTCGTCGTT (SEQ ID NO:12).

20          The nucleic acid sequences of the invention are also useful for stimulating B cell proliferation. Specific, but nonlimiting examples of such sequences include:

TCCTGTCGTTTCCTTGTCGTT (SEQ ID NO:13),  
TCCTGTCGTTTTTTGTCGTT (SEQ ID NO:14),  
TCGTCGCTGTCTGCCCTTCTT (SEQ ID NO:15),  
25       TCGTCGCTGTTGTCGTTTCTT (SEQ ID NO:16),  
TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:6),  
TCGTCGTTGTCGTTTGTCGTT (SEQ ID NO:8) and  
TGTCGTTGTCGTTGTCGTT (SEQ ID NO:11).

Preferred CpG ODN can effect at least about 500 pg/ml of TNF- $\alpha$ , 15 pg/ml IFN- $\gamma$ , 70 pg/ml of GM-CSF 275 pg/ml of IL-6, 200 pg/ml IL-12, depending on the therapeutic indication. These cytokines can be measured by assays well known in the art. The ODNs listed above or other preferred CpG ODN can effect at least about 10%, more preferably at least about 15% and most preferably at least about 20% YAC-1 cell specific lysis or at least about 30%, more preferably at least about 35%, and most preferably at least about 40% 2C11 cell specific lysis, in assays well known in the art.

An "antigenic polypeptide" is any polypeptide that can, under appropriate conditions, induce an immune response. Antigenic polypeptides include, but are not limited to, viral proteins, or fragments thereof. Minor modifications of the primary amino acid sequences of a viral polypeptide may also result in a polypeptide which have substantially equivalent antigenic activity as compared to the unmodified counterpart polypeptide. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as antigenicity still exists. One non-limiting example of an antigenic viral polypeptide is the hepatitis B surface antigen.

The term "substantially purified" as used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify viral polypeptides using standard techniques for protein purification. The substantially pure polypeptide will yield a single major band on a non-reducing polyacrylamide gel. The purity of the viral polypeptide can also be determined by amino-terminal amino acid sequence analysis.

The invention utilizes polynucleotides encoding the antigenic polypeptides. These polynucleotides include DNA, cDNA and RNA sequences which encode an antigenic polypeptide. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, polynucleotide encoding an antigenic

polypeptide may be subjected to site-directed mutagenesis, so long as the polypeptide remains antigenic.

The term "polynucleotide" or "nucleic acid sequence" refers to a polymeric form of nucleotides at least 10 bases in length. By "isolated polynucleotide" is meant a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g. a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. The term includes single and double forms of DNA.

In the present invention, the polynucleotide sequences encoding an antigenic polypeptide may be inserted into an expression vector. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the genetic sequences encoding the antigenic polypeptide. Polynucleotide sequence which encode the antigenic polypeptide can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, as start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns,



5 maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

10 By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters, are included in the invention (see *e.g.*, Bitter *et al.*, 1987, *Methods in Enzymology* 153:516-544). Promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

20 By "therapeutically effective amount" is meant the quantity of a compound according to the invention necessary to prevent, to cure or at least partially arrest symptoms in a subject. A subject is any mammal, preferably a human. Amounts effective for therapeutic use will, of course, depend on the severity of the disease and the weight and general state of the subject. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, *e.g.*, in Gilman *et al.*, eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990, each of which is herein incorporated by reference.

An oligonucleotide containing at least one unmethylated CpG can be used alone to activate the immune response or can be administered in combination with another adjuvant. An "adjuvant" is any molecule or compound which can stimulate the humoral and/or cellular immune response. For example, when the oligonucleotide containing at least one unmethylated CpG is administered in conjunction with another adjuvant, the oligonucleotide can be administered before, after, and/or simultaneously with the other adjuvant. The oligonucleotide containing at least one unmethylated CpG can have an additional efficacy (e.g., through antisense or other means) in addition to its ability to activate the immune response.

The invention further provides a method of modulating the level of a cytokine. The term "modulate" envisions the suppression of expression of a particular cytokine when it is overexpressed, or augmentation of the expression of a particular cytokine when it is underexpressed. Modulation of a particular cytokine can occur locally or systemically. It is believed that the CpG oligonucleotides do not directly activate purified NK cells, but rather render them competent to respond to IL-12 with a marked increase in their IFN- $\gamma$  production. By inducing IL-12 production and the subsequent increased IFN- $\gamma$  secretion by NK cells, the immunostimulatory nucleic acids also promote a Th1 type immune response. No direct activation of proliferation or cytokine secretion by highly purified T cells has been found. Cytokine profiles determine T cell regulatory and effector functions in immune responses.

Cytokines also play a role in directing the T cell response. Helper (CD4<sup>+</sup>) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immune system cells, including other T cells. Most mature CD4<sup>+</sup> T helper cells express one of two cytokine profiles: Th1 or Th2. Th1 cells secrete IL-2, IL-3, IFN- $\gamma$ , TNF- $\beta$ , GM-CSF and high levels of TNF- $\alpha$ . Th2 cells express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF and low levels of TNF- $\alpha$ . The Th1 subset promotes delayed-type hypersensitivity, cell-mediated immunity, and immunoglobulin class switching to IgG<sub>2a</sub>.

The Th2 subset induces humoral immunity by activating B cells, promoting antibody production, and inducing class switching to IgG<sub>1</sub> and IgE.

Several factors have been shown to influence commitment to Th1 or Th2 profiles. The best characterized regulators are cytokines. IL-12 and IFN- $\gamma$  are positive Th1 and negative Th2 regulators. IL-12 promotes IFN- $\gamma$  production, and IFN- $\gamma$  provides positive feedback for IL-12. IL-4 and IL-10 appear to be required for the establishment of the Th2 cytokine profile and to down-regulate Th1 cytokine production; the effects of IL-4 are in some cases dominant over those of IL-12. IL-13 was shown to inhibit expression of inflammatory cytokines, including IL-12 and TNF- $\alpha$  by LPS-induced monocytes, in a way similar to IL-4. The IL-12 p40 homodimer binds to the IL-12 receptor and antagonizes IL-12 biological activity; thus it blocks the pro-Th1 effects of IL-12.

This invention further provides administering to a subject having or at risk of having an virally mediated disorder, a therapeutically effective dose of a pharmaceutical composition containing the compounds of the present invention and a pharmaceutically acceptable carrier. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan.

The pharmaceutical compositions according to the invention are in general administered topically, intravenously, orally, parenterally or as implants, and even rectal use is possible in principle. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science* 249:1527-1533, 1990, which is incorporated herein by reference.

The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patient, different daily doses are necessary. Under  
5 certain circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

10 The pharmaceutical compositions according to the invention may be administered locally or systemically. By "therapeutically effective dose" is meant the quantity of a compound according to the invention necessary to prevent, to cure or at least partially arrest the symptoms of the disorder and its complications. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the patient.  
15 Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, *e.g.*, in Gilman *et al.*, eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences,  
20 17th ed., Mack Publishing Co., Easton, Pa., 1990, each of which is herein incorporated by reference.

The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that  
25 might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

## EXAMPLES

Here we evaluate the use of CpG ODN as an adjuvant for immunization of mice against hepatitis B virus surface antigen (HbsAg) given as a recombinant protein or expressed *in vivo* from a DNA vaccine.

5

Compared with the recombinant protein vaccine alone, addition of either CpG ODN or alum alone resulted in a 10-100 fold increase in the level of antibodies against HBsAg (anti-HBs). However when used together, these two adjuvants resulted in 500-1000 times higher levels of anti-HBs, indicating a strong synergistic response. Immunization with HBsAg alone or with alum resulted in a strong Th2-type response with almost all IgG being of the IgG1 isotype. CpG ODN induced a high proportion of IgG2a, indicative of a Th1-type response, even in the presence of alum.

10

DNA "vaccines" also induce potent Th1-type immune responses and this is likely due in large part to the presence of CpG motifs in the bacterially-derived plasmid DNA. We show here that responses are further augmented by the addition of CpG ODN to the DNA vaccine. The DNA vaccine but not the protein subunit vaccine was able to induce anti-HBs in mice injected on the day of birth. A combination approach of DNA prime and protein boost appears to be particularly effective for vaccination purposes, although a sufficient period (>2 weeks) must elapse before a boosting response is seen. These studies demonstrate that the addition of CpG ODN to protein or DNA vaccines is a valid new adjuvant approach to improve efficacy.

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## MATERIALS AND METHODS

### 25 Animals

Experiments on adult mice were carried out using female BALB/c (H-2<sup>d</sup>, good responder), C57BL/6 (B10, H-2<sup>b</sup>, fair responder) and B10.S (H-2<sup>s</sup>, MHC-restricted hypo-responder to HBsAg) mice (Charles River, Montreal, QC) at 6-8 weeks of age. Mice with class II MHC deficiency (C2D, H-2<sup>b</sup>, GenPharm, Mountain View, CA) due to gene knockout were used as a model of a non-responder.

30

Newborn mice were obtained through breeding male and female BALB/c mice (Charles River) in our own animal facility (Loeb Research Institute, Ottawa Civic Hospital, Ottawa, ON). Pregnant females were monitored daily to ensure accurate recording of the date of birth. Both male and female neonates were used for immunization.

5

#### **HbsAg subunit vaccination of mice**

The subunit vaccine consisted of HBsAg (ay subtype) which had been produced as a recombinant protein in yeast cells (Medix Biotech #ABH0905). This was diluted in saline for use without adjuvant. HBsAg was also formulated with alum and/or CpG ODN as  
10 adjuvant. HBsAg protein was mixed with aluminum hydroxide (Alhydrogel 85, [Al<sub>2</sub>O<sub>3</sub>], Superfos Biosector, Vedbaek, Denmark) in the same ratio of 25 mg Al<sup>3+</sup> per mg protein as used in the commercial vaccines (i.e., 2.5 l 2% Al<sub>2</sub>O<sub>3</sub> per g HBsAg). The protein and alum were mixed with a vortex and then left on ice for at least 30 minutes prior to use to allow the protein to adsorb onto the Al<sub>2</sub>O<sub>3</sub>. This solution was mixed again immediately prior to  
15 injection by drawing up into the syringe 3-5 times.

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For groups treated with CpG ODN, an appropriate volume of synthetic oligodeoxynucleotide (ODN #1826) of the sequence TCCATGACGTTCTGACGTT synthesized with a phosphorothioate backbone (Oligos Etc. & Oligo Therapeutics, Wilsonville, OR) was added alone or with alum to HBsAg on the day of injection. Adult mice received a single intramuscular (IM) injection into the left tibialis anterior (TA) muscle of 1 or 2 g HBsAg, without or with adjuvant (alum and/or CpG ODN), in 50 l vehicle. When CpG DNA was added, each animal received a total of 1, 10, 100 or 500 g ODN. Newborn mice were immunized within 24 hours of birth or 7 days after birth by bilateral injection of a total of  
1 g HBsAg into the posterior thigh muscles (2 x 10 l @ 0.05 mg/ml). All injections were carried out with a 0.3 ml insulin syringe which has a fused 29G needle (Becton Dickenson, Franklin Lakes, NJ). For injection of adults, the needle was fitted with a collar of polyethylene (PE) tubing to limit penetration of the needle to about 3 mm. All intramuscular injections were carried out through the skin (unshaved) and under general anesthesia  
(Halothane, Halocarbon Laboratories, River Edge, NJ).

**DNA-based immunization of mice**

Mice were immunized against HBsAg using plasmid constructs encoding the major protein (S) of the HBV envelope. The plasmid pCP10, containing two copies of the HBV genome (*ayw* subtype; GeneBank reference HPBAYW) as a head-to-tail fusion (Dubois *et al.*, 1980), was the source of the envelope coding sequences and the 3' untranslated sequences which include the viral polyadenylation signal. A 1.9 kb *XhoI-BglII* restriction fragment from pCP10 (containing the S coding sequences) was cloned into the corresponding sites of a modified p Bluescript SK vector containing extra restriction sites in the polylinker (kindly provided by Dr. Shahragim Tajbakhsh, Pasteur Institute). A *KpnI-BssHII* restriction fragment was then removed and cloned into the pRc/CMV expression vector (Invitrogen) using the *KpnI* site of the polylinker and the *BssHII* site within the neomycin gene of the vector. This cloning step places the envelope sequences under the control of the CMV promoter and removes the bovine growth hormone polyadenylation sequences, the fl origin, the SV40 promoter and origin, and most of the neomycin gene. An SV40 polyadenylation signal from the pRc/CMV vector is found after the transcribed HBV sequences. This construct has been described previously (Davis *et al.*, 1993) and is designated here as pCMV-S.

DNA was purified on Qiagen anion-exchange chromatography columns (Qiagen GmbH, Hilden, Germany). This method, which yields predominantly supercoiled, double-stranded, closed circular DNA, results in virtually no contamination with chromosomal DNA, RNA or protein and very low contamination with endotoxin. The DNA was resuspended in sterile saline (0.15M NaCl, BDH) and the concentration of DNA was calculated based on absorbance of ultraviolet light (OD 260). The final concentration was adjusted to 0.1 to 1 mg/ml and the DNA solutions were stored at -20C until required for injection.

Direct gene transfer in adult mice was carried out by unilateral or bilateral IM injection into the TA muscle of DNA in 50 l such that each animal received a total of 1, 10 or 100 g DNA. Newborn mice received a total of 10 g DNA by bilateral injection into the

posterior thigh muscles ( $2 \times 10^1$  @ 0.5 mg/ml). Injections were with a 0.3 ml insulin syringe which has a fused 29G needle, and for injection of adults the needle was fitted with a collar of PE tubing to limit penetration of the needle to about 2 mm. All intramuscular injections were carried out (through shaved skin for adults) under general anesthesia (Halothane)

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## Experimental groups

### *Comparison of alum and CpG ODN as adjuvant. with HBsAg subunit vaccine*

Six groups of adult BALB/c mice ( $n=10$ ) were injected with 1 g HBsAg (i) alone,  
10 (ii) mixed with alum, (iii) mixed with 100 g CpG ODN, or (iv, v, vi) mixed with both alum and 10, 100 or 500 g CpG ODN. These mice were bled at 1, 2, 3 and 4 weeks after immunization and the plasma was assayed for anti-HBs. Groups of adult C57BL/6 (B10), B10.S and C2D mice ( $n=5$ ) were injected 1 g HBsAg (i) alone, (ii) mixed with alum, (iii, iv, v) mixed with 1, 10 or 100 g CpG ODN, or (vi, vii) mixed with both alum and 10 or 100 g  
15 CpG ODN. Each animal was boosted by the identical procedure at 6 weeks. The mice were bled at 1, 2, 4, 6 and 8 weeks after immunization and the plasma was assayed for anti-HBs.

### *Use of CpG ODN as adjuvant. with HBsAg-expressing DNA vaccine*

Three groups of adult BALB/c mice ( $n=10$ ) were injected with a total of 10 g  
20 pCMV-S DNA alone or with 100 or 500 g CpG ODN added, divided between two injection sites. Groups of hypo-responder (B10.S) and congenic (B10) mice ( $n=10$ ) were immunized with 50 g pCMV-S DNA divided between two sites.

### *Immunization of neonates with subunit or DNA vaccine*

25 Groups of newborn BALB/c mice ( $n=10$ ) aged <24 hours or 7 days were injected with a total of 1 g HBsAg with alum or with 10 g pCMV-S DNA. Plasma was obtained at 4, 8, 12 and 16 weeks for assay of anti-HBs.



*Combined DNA prime with protein boost*

Five groups of BALB/c mice (n=10) were first immunized with a single injection into the left TA of 10 g pCMV-S, and received (i) no other treatment, or received 2 g pure HBsAg (no adjuvant) (ii) at the same time at the same site, (iii) at the same time and at a different site (left quadriceps), (iii) 2 weeks later at the different site or (iv) 8 weeks later at the different site. Mice were bled at 1, 2, 4, 6, 8, 12, 20 and 24 weeks.

**Evaluation of immune response to HbsAg**

Heparinized blood was collected by retrobulbar puncture of lightly anaesthetized mice as described elsewhere (Michel *et al.*, 1995). Plasma was recovered by centrifugation (7 min @ 13,000 rpm). Antibodies specific to HBsAg in plasma were detected and quantified by end-point dilution ELISA assay (in triplicate) on individual samples. Ten-fold serial dilutions of plasma were first added to 96-well microtiter plates with a solid phase consisting of plasma-derived HBsAg particles (100 l/well of HBsAg  $\alpha\gamma$  subtype at 1 g/ml, coated overnight at RT) and incubated for 1 hr at 37C. The bound antibodies were then detected by incubation for 1 hr at 37C with HRP-conjugated goat anti-mouse IgG, IgM, IgG1 or IgG2a (1:4000 in PBS-Tween, 10% FCS; 100 l/well, Southern Biotechnology Inc., Birmingham, AL), followed by incubation with OPD solution (100 l/well, Sigma, St. Louis, MO) for 30 minutes at RT in the dark. The reaction was stopped by the addition of sulfuric acid (50 l of 4N H<sub>2</sub>SO<sub>4</sub>). End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of non-immune plasma with a cut-off value of 0.05. Anti-HBs titers were expressed as group means of individual animal values, which were themselves the average of triplicate assays.

## RESULTS

### **CpG ODN versus alum as adjuvant for HBV subunit vaccine**

#### *Strength and kinetics of humoral response*

5           Immunization of BALB/c mice with HBsAg alone elicited only low titers of anti-HBs (<100) by 4 weeks. These titers were 10-fold higher with the addition of alum as adjuvant, 60-fold higher with CpG ODN and more than 500-fold higher with both alum and CpG ODN (FIG. 1). When combined with alum, there is a dose-response for CpG ODN with the best results being obtained with an intermediate dose (100 g) and similar somewhat  
10 poorer results being obtained with lower and higher doses (10 or 500 g) (FIG. 2). Nevertheless, all doses of CpG ODN greatly improved the titers compared to alum alone.

          In the C57BL/6 strain of mouse, antibody titers 4 weeks after HBsAg prime were about 10-times lower than those seen in BALB/c mice, but when boosted at 6 weeks rose to  
15 similar levels within 2 weeks. When used alone, alum and CpG ODN (100 g) each augmented the humoral response about 100-fold but when used together increased titers about 1000-fold. A dose-response for CpG ODN was also noted in these mice (in the absence of alum) with 100 g being superior to either 10 or 1 g, although all doses had an adjuvant effect (FIG. 4).

20           In the hypo-responder B10.S mice no anti-HBs antibodies were detected with HBsAg alone and low titers were obtained if either CpG ODN or alum are added. The use of alum and CpG together gives the best result although the synergy is less evident than in the BALB/c and C57BL/6 mice (FIG. 5). Non-responder (C2D) mice have no detectable  
25 anti-HBs after immunization with HBsAg alone. There are low levels of IgM with addition of alum and this is increased 4-fold with further addition of CpG ODN. There is essentially no detectable IgG after injection of HBsAg + alum, but low titers with both alum and CpG ODN (FIG. 6). Treatment with CpG DNA was well tolerated by all mice, even those receiving the 500 g dose. There was no apparent ruffling of fur, diarrhea or other signs of  
30 toxicity.

*Th1 versus Th2 responses*

Immunization with either HBsAg alone or with alum induces a predominantly Th2-type humoral response with almost all (>99%) antibodies being of the IgG1 isotype. CpG ODN induces significantly more IgG2a antibodies, which are indicative of a Th1-type response, although IgG1 still predominate. Remarkably, the combination of alum and CpG ODN induces about ten-times more IgG2a than IgG1, indicating a real shift from Th2 to Th1 (FIG. 7).

**CpG ODN as adjuvant to HBV DNA vaccine**

DNA vaccines induced higher levels of anti-HBs more rapidly than did HBsAg, even when alum was included (compare FIG.s 1 and 8). Addition of CpG ODN to the pCMV-S DNA vaccine increased anti-HBs titers a further five-fold by 4 weeks. The 500 g dose was slightly better than the 100 g dose (FIG. 8). The DNA vaccine was also superior to the HBsAg subunit vaccine in hypo-responder mice. A single injection of DNA induced earlier appearance of anti-HBs, and these reached higher titers than with two doses of protein given at 0 and 4 weeks (FIG. 9).

**Antigen versus DNA-based immunization of neonates**

Mice immunized with HBsAg plus alum on the day of birth had no detectable anti-HBs even up to 16 weeks later. In contrast, those injected with the DNA vaccine had low levels of anti-HBs by 4 weeks and a good titer ( $10^3$ ) by 16 weeks. Immunization of 7 day old mice with either DNA or protein induced anti-HBs, although these appeared much earlier and were much higher with the DNA vaccine. In fact, the DNA vaccine at one day was superior to the protein vaccine given at 7 days (FIG. 10).

**Combined DNA- and antigen-based immunization**

Co-administration of pure recombinant HBsAg and the DNA vaccine at either the same or different sites did not significantly improve titers of anti-HBs over those induced by the DNA vaccine alone. Nor was a boosting response seen when the HBsAg protein was given two weeks after the DNA vaccine. However, administration of the protein vaccine

8 weeks after the DNA vaccine gave a strong boosting response with titers increasing more than 10-fold over those with the DNA vaccine alone (FIG. 11).

## DISCUSSION

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### **CpG ODN versus alum as adjuvant with HBV subunit vaccine in mice**

CpG ODN is as good as or superior to alum when each is used alone as adjuvant with the HBsAg subunit vaccine in mice. This indicates that CpG ODN could be used to replace alum in vaccine formulations, which could be desirable to avoid associated  
10 side-effects due to local irritation in the muscle. Furthermore, for certain live-attenuated or multivalent vaccines, it is not possible to use alum which through chemical interactions interferes with the efficacy of the vaccine. This should not occur with CpG ODN.

Of even greater interest is the strong synergistic response when CpG ODN and alum  
15 are used together to adjuvant the HBsAg subunit vaccine. In humans, this could result in a much higher proportion of individuals attaining protective titers of anti-HBs after two or possibly even one dose of vaccine. Furthermore, protective titers should be reached more quickly and this would be beneficial for immunization in endemic areas. There is a fairly weak dose response to CpG ODN whether or not alum is present, indicating that a wide  
20 range of CpG ODN could be useful to adjuvant vaccines in humans.

### **CpG ODN induces Th1 response even in presence of alum**

Aluminum hydroxide (alum) is currently the only adjuvant approved for human use. An important disadvantage of alum is that it induces a Th2- rather than a Th1-type immune  
25 response, and this may interfere with induction of CTL. Indeed, in mice immunized with recombinant HBsAg, the addition of alum selectively blocked activation of CD8<sup>+</sup> CTL (Schirmbeck *et al.*, 1994). Although not essential for protective immunity against HBV, CTL may nevertheless play an important role. For example, a lack of HBV-specific CTL is thought to contribute to the chronic carrier state. In contrast, one of the primary advantages  
30 of CpG DNA over alum as an adjuvant is the Th1-bias of the responses and thus the

possibility to induce CTL. A striking finding from the present study is that CpG can completely counteract the Th2-bias of alum when the two adjuvants are delivered together. This could allow one to capitalize on the strong synergistic action of the two adjuvants on the humoral response while still allowing CTL.

5 The use of alum has been linked to Th2-type diseases. The much higher prevalence of asthma (another Th2-type disease) in more highly developed nations may be linked to the high hygiene level and rapid treatment of childhood infections (Cookson and Moffatt, 1997). Early exposure to bacterial DNA (and immunostimulatory CpG motifs) pushes the immune  
10 system away from Th2- and towards a Th1-type response and this may account for the lower incidence of asthma in less developed countries, where there is a much higher frequency of upper respiratory infections during childhood. Addition of CpG ODN as adjuvant to all pediatric vaccines could re-establish a Th1-type response thereby reducing the incidence of asthma.

#### 15 CpG dinucleotides and DNA vaccines

More recently however, it has been shown that the presence of unmethylated CpG motifs in the DNA vaccines is essential for the induction of immune responses against the antigen, which is expressed only in very small quantities (Sato *et al.*, 1996). As such, the  
20 DNA vaccine provides its own adjuvant in the form of CpG DNA. Since single-stranded but not double-stranded DNA can induce immunostimulation *in vitro* (Krieg *et al.*, unpublished observation), the CpG adjuvant effect of DNA vaccines *in vivo* is likely due to oligonucleotides resulting from plasmid degradation by nucleases. Only a small portion of the plasmid DNA injected into a muscle actually enters a myofiber and is expressed, the  
25 majority of the plasmids is degraded in the extracellular space.

#### CpG and HBV vaccines for human use

##### *Prophylactic vaccine*

Fewer than 20% of healthy individuals attain protective levels of anti-HBs  
30 (10 mIU/ml) after a single dose of subunit HBV vaccine and only 60-70% reach this level

after two doses. Thus, three doses (usually given at 0, 1 and 6 months) are required to seroconvert >90% of vaccinated individuals. The three dose regime is frequently not completed owing to poor patient compliance, and in endemic areas, protective levels may not be induced quickly enough. Thus there is a need for a prophylactic vaccine that can induce protective immunity more quickly and with fewer doses. This might be possible with the addition of CpG ODN as an adjuvant to the subunit vaccine. Another possibility is an HBsAg-expressing DNA vaccine, which could be optimized by addition of CpG dinucleotide motifs. DNA vaccines would offer additional advantages such as relatively low cost and ease of manufacturing, and heat-stability which circumvents the requirement for a cold-chain.

Neonates born in endemic areas require particularly rapid induction of strong HBV-specific immunity owing to the high rate of chronicity resulting from infection at a young age. Without immunoprophylaxis, 70-90% of infants born to mothers positive for both HBsAg and the e antigen (HBeAg) become infected and almost all of these become chronic carriers (Stevens *et al.*, 1987). Even when vaccinated with a four dose regime of the HBV subunit vaccine commencing on the day of birth, 20% of such infants became chronically infected and this was reduced to only 15% if they were also given HBV-specific immunoglobulin (Chen *et al.*, 1996). Subunit or DNA vaccines with CpG adjuvant should reduce this further owing to a more rapid appearance and higher titers of anti-HBs antibodies and the induction of HBV-specific CTL, which could help clear virus from the liver of babies infected *in utero*, and which likely account for most of the failures with neonatal vaccination. DNA vaccines could be particularly effective if coupled with a protein boost.

#### *Non-responders and hypo-responders*

Between 5 and 10% of individuals are non-responders or hypo-responders to the subunit HBsAg vaccine. This may be MHC-restricted (Kruskall *et al.*, 1992) and is thought to result from a failure to recognize T-helper epitopes. In certain immunocompromised individuals (e.g., kidney dialysis patients, alcoholics) the rate of non-response can approach

50%. In the present study, alum plus CpG ODN gave higher anti-HBs titers than alum alone in a strain of mice which has MHC-restricted hypo-responsiveness to HBsAg, thought to result in a failure to recognize T-helper epitopes. CpG ODN also overcame non-response in mice genetically incapable of providing T-help owing to an absence of class II MHC (Milich, 1988). These results support the *in vitro* finding that CpG ODN drives the T cell independent activation of B cells. Use of CpG DNA as an adjuvant may increase the response rate to HBsAg in humans. A link between MHC phenotype and non-responsiveness to HBsAg has been demonstrated in humans (Kruskall *et al.*, 1992).

#### Chronic carriers of HBV

HBV chronicity results in 10-15% of individuals infected as adolescents or adults, but 90-95% for those infected (either vertically or horizontally) as infants. HBV chronicity eventually leads to cirrhosis and increased risk of hepatocellular carcinoma and an estimated one million people die each year from HBV-related liver disease. Persistent HBV infection of the liver results when acute infection fails to launch an appropriate immune response to clear the virus. Such chronic carriers have circulating HBsAg and HBV core antigen (HBcAg/HBeAg) without specific immunity. It is thought that the absence of HBV-specific CTL may contribute to the establishment and maintenance of the chronic carrier state. Indeed, many previously infected individuals, even years after clinical and serological recovery, have traces of HBV in their blood and HBV-specific CTL that express activation markers indicative of recent contact with antigen (Rehermann *et al.*, 1996). These results suggest that sterilizing immunity may not occur after HBV infection and that chronic activation of CTL is responsible for keeping the virus under control.

There is currently no cure for the HBV chronic infection. Interferon is used currently but this cures only 10-20% of treated individuals (Niederau *et al.*, 1996). Anti-viral drugs (e.g., lamivudine) can reduce circulating virus to undetectable levels, however these return to pretreatment levels if the drug is stopped. Each of these types of treatment is also expensive and has certain undesirable side-effects.

The possibility to induce a strong Th1-type response with CpG ODN added to a subunit vaccine may help overcome the chronic carrier state. For this application, it might be desirable to include additional B and T cell epitopes encoded by other domains of the HBV envelope protein (e.g., pre-S1 and pre-S2). Since the pre-S1 polypeptide may prevent secretion, it might be desirable to encode a truncated version of this such as that described by Li *et al.* (1994) with only amino acids 21-47 which include the hepatocyte receptor-binding site and induce anti-preS1 immune responses yet still maintain particle secretion. Repeated doses of a subunit vaccine containing the middle HBV envelope protein (preS2 + S) reduced viral replication in 50% of vaccinated chronic carriers (Pol *et al.*, 1993). Addition of CpG ODN would presumably improve these results through its strong Th1 bias.

A DNA vaccine might also prove very effective as a therapeutic vaccine for HBV chronic carriers. We have previously shown that an HBsAg-expressing DNA vaccine could break tolerance to HBsAg in transgenic mice expressing HBsAg in the liver from before birth (Mancini *et al.*, 1996). This response appears to be mediated by T cells via a non-lytic mechanism. Addition of CpG dinucleotide motifs that preferentially induce Th1 cytokines and strong CTL responses could further improve DNA vaccines for application to HBV chronic carriers.

## CONCLUSION

ODN containing CpG dinucleotides, in the proper base context to cause immune activation, are useful as an adjuvant to protein vaccines (whole pathogen or subunit). The CpG ODN could be used alone or in combination with alum. Used alone, it will allow the possibility to adjuvant vaccines that cannot be mixed with alum (e.g., live attenuated pathogens, multivalent vaccines). Used together, it will capitalize on the synergistic effect to induce very potent immune responses, yet still maintain the Th1 bias of CpG DNA. CpG dinucleotides also act to adjuvant DNA vaccines. Additional CpG given as ODN or cloned into the plasmid vector could further augment immune responses.



With respect to vaccines against HBV, CpG ODN could be added as an adjuvant to recombinant HBsAg subunit vaccines, either alone or in combination with alum, or can be cloned into an HBsAg-expressing DNA vaccine. These improved vaccines can (i) induce higher titers more quickly and reduce the number of doses required to induce protective immunity from three to two, (ii) overcome hypo- or non- responsiveness to HBsAg, (iii) control the chronic carrier state through induction of CTL, and (iv) induce rapid and stronger immunity in neonates in HBV endemic areas.

## REFERENCES

- Chen D.S. *et al.* (1996). *Cancer Causes & Control* 7: 305-311.
- Cookson, W.O.C.M. & Moffatt, M.F. (1997). *Science* 275: 41-42.
- Cowdery, J.S. *et al.* (1996). *J. Immunol.* 156: 4570.
- Davis, H.L. *et al.* (1993). *Human Molec. Genet.* 2: 1847-1851.
- Davis, H.L. *et al.* (1995). *Human Gene Ther.* 6: 1447-1456.
- Davis, H.L. *et al.* (1996). *Proc. Natl. Acad. Sci. USA* 93: 7213-7218.
- Davis, H.L. *et al.* (1996). *Vaccine* 14: 910-915.
- Davis, H.L. *et al.* (1997). *Gene Ther.* (in press).
- Davis, H.L. & Brazolot Millan, C.L. (1997). *Blood Cell Biochem.* (in press)
- Donnelly, J.J. *et al.* (1996). *Life Sciences.* 60: 163-172.
- Dubois, M.-F. *et al.* (1980). *Proc. Natl. Acad. Sci. USA* 77: 4549-4553.
- Ellis, R. W. (Ed.) (1993). *Hepatitis B Vaccines in Clinical Practise*. New York: Marcel-Dekker.
- Halpern, M.D. *et al.* (1996). *Cell. Immunol.* 167: 72.
- Klinman, D.M. *et al.* (1996). *Proc. Natl. Acad. Sci. USA* 93: 2879-2883.
- Krieg, A.M. *et al.* (1995). *Nature* 374: 546-549.
- Kruskall, M.S. *et al.* (1992). *J. Exp. Med.* 175: 495-502.
- Li *et al.* , (1994). *J. Gen. Virol.* 75: 3673-3677.
- Mancini, M. *et al.* (1996). *Proc. Natl. Acad. Sci. USA* 93: 12496-12501.

Michel, M.-L. *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81: 7708-7712.

Michel, M.-L. *et al.* (1995). *Proc. Natl. Acad. Sci. USA* 92: 5307-5311.

Milich, D.R. (1988). *Immunol. Today* 9: 380-386.

Niederau *et al.* (1996). *New Eng. J. Med.* 334: 1422-1427.

Pol, S. *et al.* (1993). *C. R. Acad. Sci. (Paris)* 316: 688-691.

Rehermann, B. *et al.* (1996). *Nature Med.* 2: 1104-1108.

Sato, Y., *et al.* (1996). *Science* 273: 352-354.

Schirmbeck, R. *et al.* (1994). *J. Immunol.* 152: 1110-1119.

Stevens, C.E. *et al.* (1987). *J. Am. Med. Assoc.* 257: 2612-2616.

Vogel, F.R. & Sarver, N. (1995). *Clin. Microbiol. Rev.* 8: 406-410.

- 1 1. A method of inducing an immune response in a subject, said method comprising:  
2 administering to the subject a therapeutically effective amount of nucleic acid  
3 encoding an antigenic polypeptide, and a therapeutically effective amount of an  
4 oligonucleotide containing at least one unmethylated CpG dinucleotide.
- 1 2. The method of claim 1, wherein the oligonucleotide is from 8-30 bases in length.
- 1 3. The method of claim 1, wherein the subject is human.
- 1 4. The method of claim 1, wherein said nucleic acid encoding an antigenic protein and an  
2 effective amount of an oligonucleotide containing at least one unmethylated CpG  
3 dinucleotide are administered in a vector.
- 1 5. The method of claim 1, wherein said nucleic acid encoding an antigenic protein encodes a  
2 viral antigen.
- 1 6. The method of claim 5, wherein said viral antigen is a hepatitis viral antigen.
- 1 7. The method of claim 6, wherein said hepatitis viral antigen is a hepatitis B virus surface  
2 antigen.
- 1 8. The method of claim 1, further comprising administering an adjuvant.
- 1 9. The method of claim 8, wherein the adjuvant is aluminum hydroxide.
- 1 10. The method of claim 1, wherein the oligonucleotide has a formula:  
2  $5'N_1X_1CGX_2N_23'$  (SEQ ID NO:1)  
3 wherein at least one nucleotide separates consecutive CpGs;  $X_1$  is adenine, guanine, or  
4 thymidine;  $X_2$  is cytosine or thymine,  $N$  is any nucleotide and  $N_1 + N_2$  is from about 0-26  
5 bases.

- 1 11. The method of claim 10, wherein  $N_1$  and  $N_2$  do not contain a CCGG quadmer or  
2 more than one CGG trimer; and the nucleic acid sequence is from about 8-30  
3 bases in length.
- 1 12. The method of claim 1, wherein the oligonucleotide has a formula:  
2 5'  $N_1X_1X_2CGX_3X_4N_2$  3' (SEQ ID NO:2)  
3 wherein at least one nucleotide separates consecutive CpGs;  $X_1X_2$  is selected from the  
4 group consisting of GpT, GpG, GpA, ApT and ApA;  $X_3X_4$  is selected from the group  
5 consisting of TpT or CpT; N is any nucleotide and  $N_1 + N_2$  is from about 0-26 bases.
- 1 13. The method of claim 12, wherein  $N_1$  and  $N_2$  do not contain a CCGG quadmer or  
2 more than one CCG or CGG trimer; and the nucleic acid sequence is from about  
3 8-30 bases in length.
- 1 14. The method of claim 12, wherein said oligonucleotide is 5'-  
2 TCCATGACGTTCTGACGTT-3' (SEQ ID NO:3).
- 1 15. The method of claim 1, said further comprising:  
2 administering to the subject an therapeutically effective amount of the antigenic  
3 polypeptide.
- 1 16. A method for treating a subject having or at risk of having a viral-mediated  
2 disorder, comprising administering to the subject a therapeutically effective  
3 amount of a nucleic acid encoding an antigenic polypeptide and an effective  
4 amount of an oligonucleotide containing at least one unmethylated CpG  
5 dinucleotide.
- 1 17. The method of claim 16, wherein the oligonucleotide is from 8-30 bases in length.
- 1 18. The method of claim 16, wherein the subject is human.

- 1 19. The method of claim 16, wherein said nucleic acid encoding an antigenic protein  
2 and an effective amount of an oligonucleotide containing at least one  
3 unmethylated CpG dinucleotide are administered in a vector.
- 1 20. The method of claim 16, said further comprising administering to the subject an  
2 therapeutically effective amount of the antigenic polypeptide .
- 1 21. The method of claim 16, wherein said nucleic acid encoding an antigenic  
2 polypeptide encodes a viral antigen.
- 1 22. The method of claim 20, wherein said viral antigen is a hepatitis antigen.
- 1 23. The method of claim 22, wherein said hepatitis antigen is a hepatitis B surface  
2 antigen.
- 1 24. The method of claim 16, further comprising administering an adjuvant.
- 1 25. The method of claim 24, wherein the adjuvant is aluminum hydroxide.
- 1 26. The method of claim 16, wherein the oligonucleotide has a formula:  
2  $5'N_1X_1CGX_2N_23'$  (SEQ ID NO:1)  
3 wherein at least one nucleotide separates consecutive CpGs;  $X_1$  is adenine, guanine, or  
4 thymidine;  $X_2$  is cytosine or thymine, N is any nucleotide and  $N_1 + N_2$  is from about 0-26  
5 bases.
- 1 27. The method of claim 26, wherein  $N_1$  and  $N_2$  do not contain a CCGG quadmer or  
2 more than one CGG trimer; and the nucleic acid sequence is from about 8-30  
3 bases in length.

- 1 28. The method of claim 16, wherein the oligonucleotide has a formula:  
2 5' N<sub>1</sub>X<sub>1</sub>X<sub>2</sub>CGX<sub>3</sub>X<sub>4</sub>N<sub>2</sub> 3' (SEQ ID NO:2)  
3 wherein at least one nucleotide separates consecutive CpGs; X<sub>1</sub>X<sub>2</sub> is selected from the  
4 group consisting of GpT, GpG, GpA, ApT and ApA; X<sub>3</sub> X<sub>4</sub> is selected from the group  
5 consisting of TpT or CpT; N is any nucleotide and N<sub>1</sub> + N<sub>2</sub> is from about 0-26 bases.
- 1 29. The method of claim 28, wherein N<sub>1</sub> and N<sub>2</sub> do not contain a CCGG quadmer or  
2 more than one CCG or CGG trimer; and the nucleic acid sequence is from about  
3 8-30 bases in length.
- 1 30. The method of claim 28, wherein said oligonucleotide is 5'-  
2 TCCATGACGTTCTGACGTT-3' (SEQ ID NO:3).
- 1 31. A method for treating a subject having or at risk of having a chronic viral  
2 infection, said method comprising administering to the subject an effective  
3 amount of a nucleic acid encoding an antigenic polypeptide and an effective  
4 amount of an oligonucleotide containing at least one unmethylated CpG  
5 dinucleotide.
- 1 32. The method of claim 31, wherein the oligonucleotide is from 8-30 bases in length.
- 1 33. The method of claim 31, wherein the subject is human.
- 1 34. The method of claim 31, said further comprising administering to the subject an  
2 therapeutically effective amount of the antigenic polypeptide .
- 1 35. The method of claim 31, further comprising administering an adjuvant.
- 1 36. The method of claim 34, wherein the adjuvant is aluminum hydroxide.
- 1 37. The method of claim 31, wherein said antigenic protein is a viral antigen.

- 1 38. The method of claim 37, wherein said viral antigen is a hepatitis viral antigen.
- 1 39. The method of claim 38, wherein said hepatitis viral antigen is a hepatitis B  
2 surface antigen.
- 1 40. The method of claim 31, wherein the oligonucleotide has a formula:  
2 5'N<sub>1</sub>X<sub>1</sub>CGX<sub>2</sub>N<sub>2</sub>3' (SEQ ID NO:1)  
3 wherein at least one nucleotide separates consecutive CpGs; X<sub>1</sub> is adenine, guanine, or  
4 thymidine; X<sub>2</sub> is cytosine or thymine, N is any nucleotide and N<sub>1</sub> + N<sub>2</sub> is from about 0-26  
5 bases.
- 1 41. The method of claim 40, wherein N<sub>1</sub> and N<sub>2</sub> do not contain a CCGG quadmer or  
2 more than one CGG trimer; and the nucleic acid sequence is from about 8-30  
3 bases in length.
- 1 42. The method of claim 31, wherein the oligonucleotide has a formula:  
2 5'N<sub>1</sub>X<sub>1</sub>X<sub>2</sub>CGX<sub>3</sub>X<sub>4</sub>N<sub>2</sub>3' (SEQ ID NO:2)  
3 wherein at least one nucleotide separates consecutive CpGs; X<sub>1</sub>X<sub>2</sub> is selected from the  
4 group consisting of GpT, GpG, GpA, ApT and ApA; X<sub>3</sub>X<sub>4</sub> is selected from the group  
5 consisting of TpT or CpT; N is any nucleotide and N<sub>1</sub> + N<sub>2</sub> is from about 0-26 bases.
- 1 43. The method of claim 42, wherein N<sub>1</sub> and N<sub>2</sub> do not contain a CCGG quadmer or  
2 more than one CCG or CGG trimer; and the nucleic acid sequence is from about  
3 8-30 bases in length.
- 1 44. The method of claim 42, wherein said oligonucleotide is  
2 5'-TCCATGACGTTTCCTGACGTT-3' (SEQ ID NO:3).



- 1     45.     A pharmaceutical composition comprising an immunostimulatory CpG  
2             oligonucleotide and a nucleic acid encoding an antigenic protein in a  
3             pharmaceutically acceptable carrier.

## AMENDED CLAIMS

[received by the International Bureau on 14 August 1998 (14.08.98);  
original claims 1-45 cancelled; new claims 1-136 added (15 pages)]

1. A method of inducing protective immune response in a subject having or at risk of having infection with an infectious organism, said method comprising administering to the subject a therapeutically effective amount of an antigen, and a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.  
5
2. The method of claim 1, wherein said infectious organism is a bacteria or a parasite.
3. The method of claim 1, wherein said infectious organism is a virus.
4. The method of claim 3, wherein said virus is a hepatitis virus.
- 10 5. The method of claim 4, wherein said hepatitis virus is a hepatitis B virus.
6. The method of claim 1, wherein the oligonucleotide is from 8-30 bases in length.
7. The method of claim 1, wherein the oligonucleotide is contained within a plasmid made with a natural phosphodiester backbone.
- 15 8. The method of claim 1, wherein the oligonucleotide is made completely or partially with a synthetic backbone.
9. The method of claim 8, wherein the oligonucleotide is made completely with a synthetic phosphorothioate backbone.
10. The method of claim 8, wherein the oligonucleotide is made with a chimeric backbone with synthetic phosphorothioate linkages at the 3' and 5' ends and natural phosphodiester linkages in the CpG-containing center.  
20

11. The method of claim 10, wherein the said chimeric oligonucleotide is made with synthetic phosphorothioate linkages for five linkages at the 3' end and two linkages at the 5' end, and with natural phosphodiester linkages in between.
- 5 12. The method of claim 1, wherein the oligonucleotide has a formula: 5'N1X1CGX2N23' (SEQ ID NO: 1) wherein at least one nucleotide separates consecutive CpGs; X1 is adenine, guanine or thymidine; X2 is cytosine or thymine; N is any nucleotide and N1 + N2 is from about 0-26 bases.
- 10 13. The method of claim 12, wherein the N1 + N2 do not contain a CCGG quadmer or more than one CGG trimer, and the nucleic acid sequence is from about 8-30 bases in length.
14. The method of claim 1, wherein the oligonucleotide has a formula: 5'N1X1X2CGX3X4N23' (SEQ ID NO: 2) wherein at least one nucleotide separates consecutive CpGs; X1X2 is selected from the group consisting of  
15 GpT, GpG, GpA, ApT and ApA; X3X4 is selected from the group consisting of TpT and CpT; N is any nucleotide and N1 + N2 is from about 0-26 bases.
15. The method of claim 14, wherein the N1 + N2 do not contain a CCGG quadmer or more than one CGG trimer, and the nucleic acid sequence is from about 8-30 bases in length.
- 20 16. The method of claim 14, wherein said oligonucleotide is 5'-TCCATGACGTTTCCTGACGTT-3' (SEQ ID NO:3).
17. The method of claim 1, wherein the antigen is a polypeptide.
18. The method of claim 17, wherein said antigenic polypeptide is from a virus.

19. The method of claim 18, wherein said viral antigen is a hepatitis viral antigen.
20. The method of claim 19, wherein said hepatitis viral antigen is a hepatitis B viral antigen.
- 5 21. The method of claim 20, wherein said hepatitis B viral antigen is a hepatitis B virus surface antigen.
22. The method of claim 1, further comprising at least one additional adjuvant.
23. The method of claim 22, wherein the additional adjuvant contains aluminum (alum).
- 10 24. The method of claim 23, wherein the aluminum-containing adjuvant is aluminum hydroxide.
25. The method of claim 1, wherein the antigen and the CpG containing oligonucleotide are administered in a delivery vector or vehicle.
26. The method of claim 1, wherein the subject is a mammal.
- 15 27. The method of claim 1, wherein the subject is a human.
28. A method of inducing an immune response in a subject, said method comprising: administering to the subject a therapeutically effective amount of nucleic acid encoding an antigenic polypeptide, and a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.
- 20

29. The method of claim 28, wherein said immune response is to prevent infection by an infectious organism.
30. The method of claim 29, wherein said infectious organism is a virus.
31. The method of claim 30, wherein said virus is a hepatitis virus.
- 5 32. The method of claim 31, wherein said hepatitis virus is a hepatitis B virus.
33. The method of claim 28, wherein said nucleic acid vector is a DNA vector
34. The method of claim 33, wherein the CpG containing oligonucleotide is cloned into said vector.
35. The method of claim 33, wherein said DNA vector is plasmid DNA.
- 10 36. The method of claim 28, wherein the CpG-containing oligonucleotide is a separate entity from the antigen-encoding nucleic acid vector.
37. The method of claim 36, wherein the CpG oligonucleotide is contained in a plasmid with a phosphodiester backbone.
38. The method of claim 36, wherein said CpG-containing oligonucleotide has  
15 a partially synthetic backbone.
39. The method of claim 38, wherein said oligonucleotide is made with a chimeric backbone with synthetic phosphorothioate linkages at the 3' and 5' ends and natural phosphodiester linkages in the CpG-containing center.

40. The method of claim 39, wherein said chimeric oligonucleotide is made with synthetic phosphorothioate linkages for five linkages at the 3' end and two linkages at the 5' end, and with natural phosphodiester linkages in between.
41. The method of claim 28, wherein the oligonucleotide has a formula:  
5 5'N1X1CGX2N23' (SEQ ID NO: 1) wherein at least one nucleotide separates consecutive CpGs; X1 is adenine, guanine or thymidine; X2 is cytosine or thymine; N is any nucleotide and N1 + N2 is from about 0-26 bases.
42. The method of claim 41, wherein the N1 + N2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from  
10 about 8-1000 bases in length.
43. The method of claim 28, wherein the oligonucleotide has a formula:  
5'N1X1X2CGX3X4N23' (SEQ ID NO: 2) wherein at least one nucleotide separates consecutive CpGs; X1X2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X3X4 is selected from the group consisting of  
15 TpT and CpT; N is any nucleotide and N1 + N2 is from about 0-26 bases.
44. The method of claim 43, wherein the N1 + N2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-1000 bases in length.
45. The method of claim 43, wherein said oligonucleotide is  
20 5'-TCCATGACGTTTCCTGACGTT-3' (SEQ ID NO:3).
46. The method of claim 28, wherein said antigen-encoding nucleic acid encodes a viral antigen.

47. The method of claim 46, wherein said viral antigen is a hepatitis viral antigen.
48. The method of claim 47, wherein said hepatitis viral antigen is a hepatitis B viral antigen.
- 5 49. The method of claim 48, wherein said hepatitis B viral antigen is a hepatitis B virus surface antigen.
50. The method of claim 28, wherein the antigen-encoding nucleic acid and the CpG-dinucleotide are administered in a delivery vector or vehicle.
51. The method of claim 28, wherein the antigen-encoding nucleic acid and the  
10 CpG-dinucleotide are administered with an additional adjuvant.
52. The method of claim 28, wherein the subject is a mammal.
53. The method of claim 28, wherein the subject is a human.
54. A method of inducing an immune response in a subject, said method  
15 comprising administering to the subject a therapeutically effective amount of nucleic acid encoding an antigenic polypeptide, a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide, and a therapeutically effective amount of an antigen.
55. The method of claim 54, wherein the CpG dinucleotide is contained in same  
20 plasmid as that encoding the antigen and this is administered mixed together with the antigen.

56. The method of claim 54, wherein said antigen-encoding and CpG dinucleotide-containing nucleic acid is given at a different site than said antigen plus CpG dinucleotide-containing oligonucleotide.
57. The method of claim 54, wherein the said antigen-encoding and CpG dinucleotide-containing nucleic acid is given at a different time from said antigen plus CpG dinucleotide-containing oligonucleotide.
58. The method of claim 57, wherein the immune response is primed by said antigen-encoding and CpG dinucleotide-containing nucleic acid and the response is boosted by said antigen plus CpG dinucleotide-containing oligonucleotide.
59. The method of claim 54, wherein said antigenic polypeptide is the same as that encoded by the antigen-encoding nucleic acid.
60. The method of claim 54, wherein said antigenic polypeptide is different than that encoded by the antigen-encoding nucleic acid.
61. A method of treating a subject having an infectious disorder that is chronic or likely to become chronic, said method comprising: administering to the subject a therapeutically effective amount of an antigen, and a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.
62. The method of claim 61, wherein said infectious disease is viral.
63. The method of claim 62 wherein said viral disease is hepatitis.
64. The method of claim 63, wherein said hepatitis disease is hepatitis B.



65. The method of claim 61, wherein the oligonucleotide is from 8-30 bases in length.
66. The method of claim 61, wherein the oligonucleotide is contained within a plasmid made with a natural phosphodiester backbone.
- 5 67. The method of claim 61, wherein the oligonucleotide is made completely or partially with a synthetic backbone.
68. The method of claim 67, wherein the oligonucleotide is made completely with a synthetic phosphorothioate backbone.
69. The method of claim 67, wherein the oligonucleotide is made with a chimeric backbone with synthetic phosphorothioate linkages at the 3' and 5' ends and  
10 natural phosphodiester linkages in the CpG-containing center.
70. The method of claim 69, wherein the oligonucleotide is made with a chimeric backbone with synthetic phosphorothioate linkages for five linkages at the 3' end and two linkages at the 5' end, and with natural phosphodiester linkages  
15 in between.
71. The method of claim 61, wherein the oligonucleotide has a formula: 5'N1X1CGX2N23' (SEQ ID NO: 1) wherein at least one nucleotide separates consecutive CpGs; X1 is adenine, guanine or thymidine; X2 is cytosine or thymine; N is any nucleotide and N1 + N2 is from about 0-26 bases.
- 20 72. The method of claim 71, wherein the N1 + N2 do not contain a CCGG quadmer or more than one CGG trimer, and the nucleic acid sequence is from about 8-30 bases in length.

73. The method of claim 61, wherein the oligonucleotide has a formula:  
5'N1X1X2CGX3X4N23' (SEQ ID NO: 2) wherein at least one nucleotide  
separates consecutive CpGs; X1X2 is selected from the group consisting of  
GpT, GpG, GpA, ApT and ApA; X3X4 is selected from the group consisting  
of TpT and CpT; N is any nucleotide and N1 + N2 is from about 0-26 bases.
74. The method of claim 73, wherein the N1 + N2 do not contain a CCGG  
quadmer or more than one CGG trimer; and the nucleic acid sequence is from  
about 8-30 bases in length.
75. The method of claim 73, wherein said oligonucleotide is  
5'-TCCATGACGTCCTGACGTT-3' (SEQ ID NO:3).
76. The method of claim 61, wherein the antigen is a polypeptide.
77. The method of claim 76, wherein said antigenic polypeptide is from a virus.
78. The method of claim 77, wherein said viral antigen is a hepatitis viral  
antigen.
79. The method of claim 78, wherein said hepatitis viral antigen is a hepatitis B  
viral antigen.
80. The method of claim 79, wherein said hepatitis B viral antigen is a hepatitis  
B virus surface antigen.
81. The method of claim 61, further comprising at least one additional adjuvant.
82. The method of claim 81, wherein the additional adjuvant contains aluminum  
(alum).

83. The method of claim 82, wherein the additional aluminum-containing adjuvant is aluminum hydroxide.
84. The method of claim 61, wherein the antigen and the CpG containing oligonucleotide are administered in a delivery vector or vehicle.
- 5 85. The method of claim 61, wherein the subject is a mammal.
86. The method of claim 61, wherein the subject is a human.
87. A method of treating a subject having an infectious disorder that is chronic or likely to become chronic, said method comprising administering to the subject a therapeutically effective amount of nucleic acid encoding an antigenic polypeptide, and a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide.
- 10
88. The method of claim 87, wherein said infectious disease is viral.
89. The method of claim 88, wherein said viral disease is hepatitis.
90. The method of claim 89, wherein said hepatitis disease is hepatitis B.
- 15 91. The method of claim 87, wherein said nucleic acid vector is a DNA vector
92. The method of claim 91, wherein the CpG containing oligonucleotide is cloned into said vector.
93. The method of claim 91, wherein said DNA vector is plasmid DNA.

94. The method of claim 87, wherein the CpG-containing oligonucleotide is a separate entity from the antigen-encoding nucleic acid vector.
95. The method of claim 94, wherein said CpG-containing oligonucleotide has a completely or partially synthetic backbone.
- 5 96. The method of claim 95, wherein said oligonucleotide is made with a chimeric backbone with synthetic phosphorothioate linkages at the 3' and 5' ends and natural phosphodiester linkages in the CpG-containing center.
97. The method of claim 96, wherein said chimeric oligonucleotide is made with synthetic phosphorothioate linkages for five linkages at the 3' end and two  
10 linkages at the 5' end, and with natural phosphodiester linkages in between.
98. The method of claim 87, wherein the oligonucleotide has a formula: 5'N1X1CGX2N23' (SEQ ID NO: 1) wherein at least one nucleotide separates consecutive CpGs; X1 is adenine, guanine or thymidine; X2 is cytosine or thymine; N is any nucleotide and N1 + N2 is from about 0-26 bases.
- 15 99. The method of claim 98, wherein the N1 + N2 do not contain a CCGG quadmer or more than one CGG trimer, and the nucleic acid sequence is from about 8-1000 bases in length.
100. The method of claim 87, wherein the oligonucleotide has a formula: 5'N1X1X2CGX3X4N23' (SEQ ID NO: 2) wherein at least one nucleotide  
20 separates consecutive CpGs; X1X2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X3X4 is selected from the group consisting of TpT and CpT; N is any nucleotide and N1 + N2 is from about 0-26 bases.

101. The method of claim 100, wherein the N1 + N2 do not contain a CCGG quadmer or more than one CGG trimer, and the nucleic acid sequence is from about 8-1000 bases in length.
102. The method of claim 100, wherein said oligonucleotide is  
5 5'-TCCATGACGTTTCCTGACGTT-3' (SEQ ID NO:3).
103. The method of claim 87, wherein said antigen-encoding nucleic acid encodes a viral antigen.
104. The method of claim 103, wherein said viral antigen is a hepatitis viral antigen.
- 10 105. The method of claim 104, wherein said hepatitis viral antigen is a hepatitis B viral antigen.
106. The method of claim 105, wherein said hepatitis B viral antigen is a hepatitis B virus surface antigen.
107. The method of claim 87, wherein the antigen-encoding nucleic acid and the  
15 CpG-dinucleotide are administered in a delivery vector or vehicle.
108. The method of claim 87, wherein the antigen-encoding nucleic acid and the CpG-dinucleotide are administered with an additional adjuvant.
109. The method of claim 87, wherein the subject is a mammal.
110. The method of claim 87, wherein the subject is a human.

111. A method of treating a subject having an infectious disorder that is chronic or likely to become chronic, said method comprising: administering to the subject a therapeutically effective amount of nucleic acid encoding an antigenic polypeptide, a therapeutically effective amount of an oligonucleotide containing at least one unmethylated CpG dinucleotide, and a therapeutically effective amount of an antigen.
- 5
112. The method of claim 111, wherein the CpG dinucleotide is contained in the same plasmid as that encoding the antigen.
113. The method of claim 112, wherein said antigen-encoding and CpG dinucleotide-containing nucleic acid is given mixed together with said antigen.
- 10
114. The method of claim 111, wherein said antigen-encoding and CpG dinucleotide-containing nucleic acid is given at a different site than said antigen plus CpG dinucleotide-containing oligonucleotide.
115. The method of claim 111, wherein the CpG-containing oligonucleotide is a separate entity from the nucleic acid encoding an antigenic polypeptide.
- 15
116. The method of claim 115, wherein said CpG-containing oligonucleotide is contained within a plasmid.
117. The method of claim 115, wherein said CpG-containing oligonucleotide is made with a completely or partially synthetic backbone.
- 20 118. The method of claim 111, wherein the said antigen-encoding and CpG dinucleotide-containing nucleic acid is given at a different time from said antigen plus CpG dinucleotide-containing oligonucleotide.

119. The method of claim 118, wherein the immune response is primed by said antigen-encoding and CpG dinucleotide-containing nucleic acid and the response is boosted by said antigen plus CpG dinucleotide-containing oligonucleotide.
- 5 120. The method of claim 111, wherein said antigenic polypeptide is the same as that encoded by the antigen-encoding nucleic acid.
121. The method of claim 111, wherein said antigenic polypeptide is different than that encoded by the antigen-encoding nucleic acid.
- 10 122. A pharmaceutical composition comprising an antigen and an oligonucleotide containing an immunostimulatory CpG motif in a pharmaceutically acceptable carrier.
123. The composition of claim 122, wherein the said antigen is a viral antigen.
124. The composition of claim 123, wherein the said viral antigen is a hepatitis viral antigen.
- 15 125. The composition of claim 124, wherein the said viral hepatitis antigen is a hepatitis B antigen.
126. The composition of claim 125, wherein the said hepatitis B antigen is a hepatitis B surface antigen.
- 20 127. A pharmaceutical composition comprising a nucleic acid encoding an antigenic protein and an oligonucleotide containing an immunostimulatory CpG motif in a pharmaceutically acceptable carrier.

128. The composition of claim 127, wherein the said antigen is a viral antigen.
129. The composition of claim 128, wherein the said viral antigen is a hepatitis viral antigen.
130. The composition of claim 129, wherein the said viral hepatitis antigen is a hepatitis B antigen.
- 5 131. The composition of claim 130, wherein the said hepatitis B antigen is a hepatitis B surface antigen.
132. A pharmaceutical composition comprising a nucleic acid encoding an antigenic protein and an antigen in a pharmaceutically acceptable carrier.
- 10 133. The composition of claim 132, wherein the said antigen is a viral antigen
134. The composition of claim 133, wherein the said viral antigen is a hepatitis viral antigen.
135. The composition of claim 134, wherein the said viral hepatitis antigen is a hepatitis B antigen.
- 15 136. The composition of claim 135, wherein the said hepatitis B antigen is a hepatitis B surface antigen.

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## Immunization of BALB/c mice against HBsAg using alum and/or CpG oligos as adjuvant

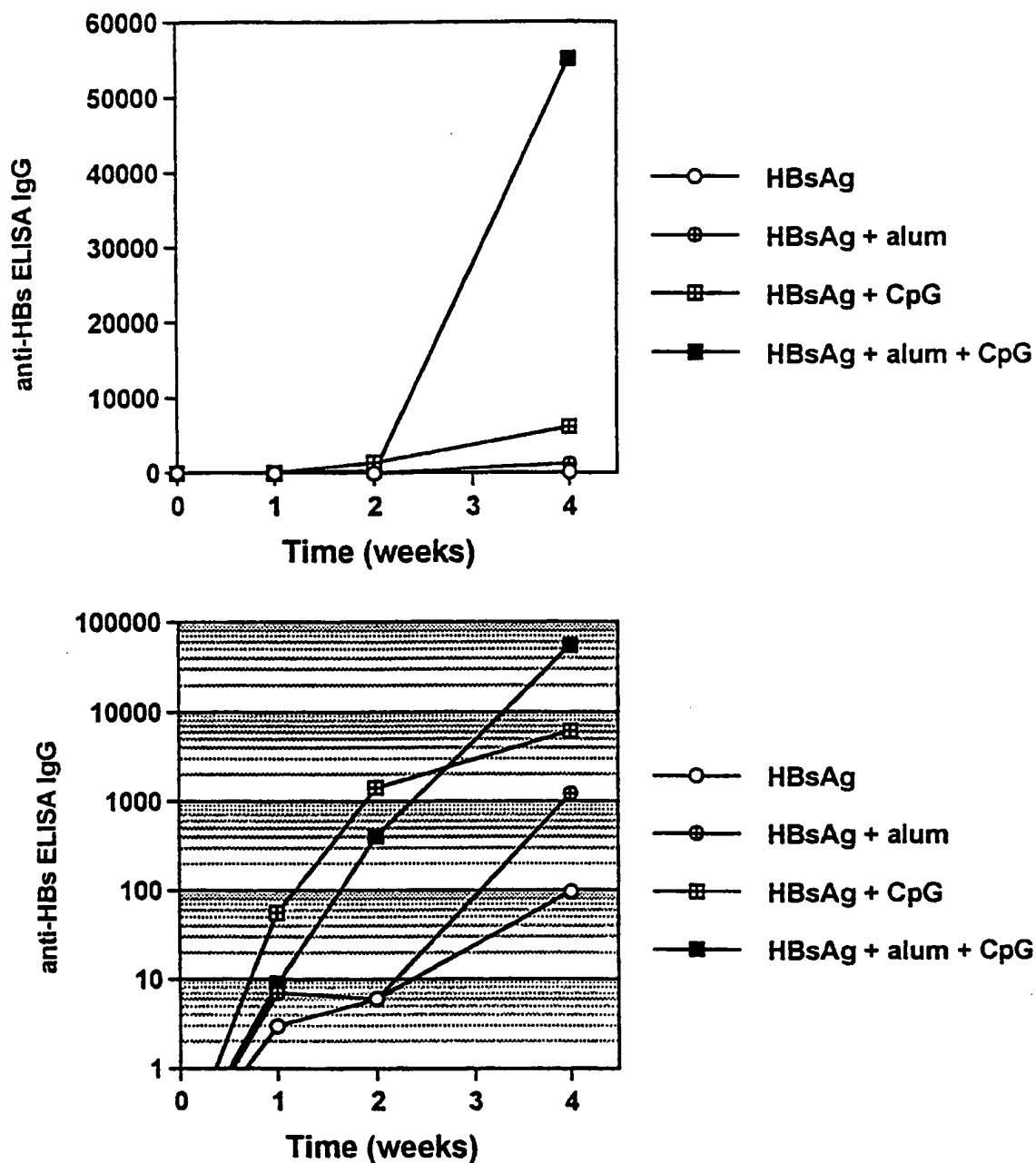
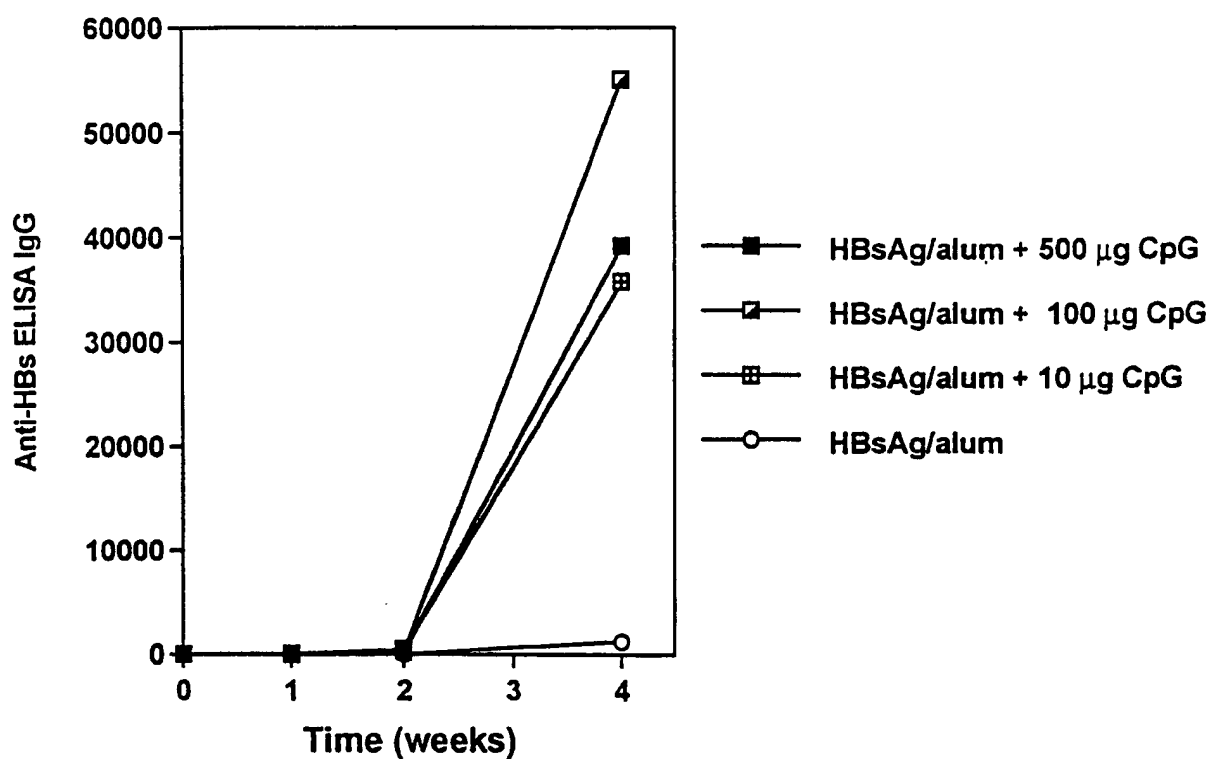


Figure 1

**Immunization of BALB/c mice with HBV vaccine**  
**Dose response for CpG oligos**



**Figure 2**

# Immunization of C57BL/6 mice against HBsAg Use of alum and/or CpG oligos as adjuvant

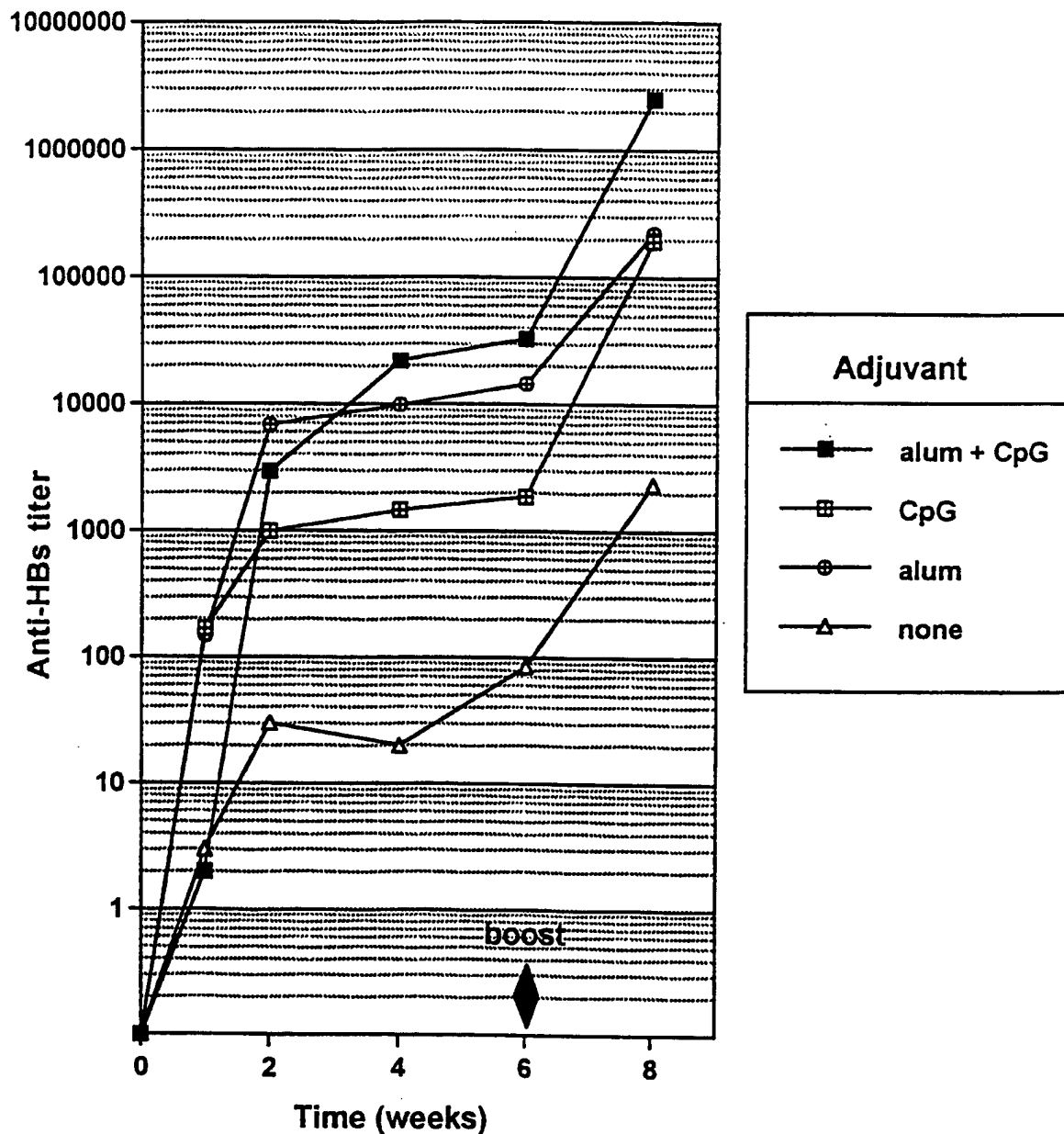


Figure 3

# Immunization of C57BL/6 mice against HBsAg Use of CpG oligos as adjuvant : Dose response

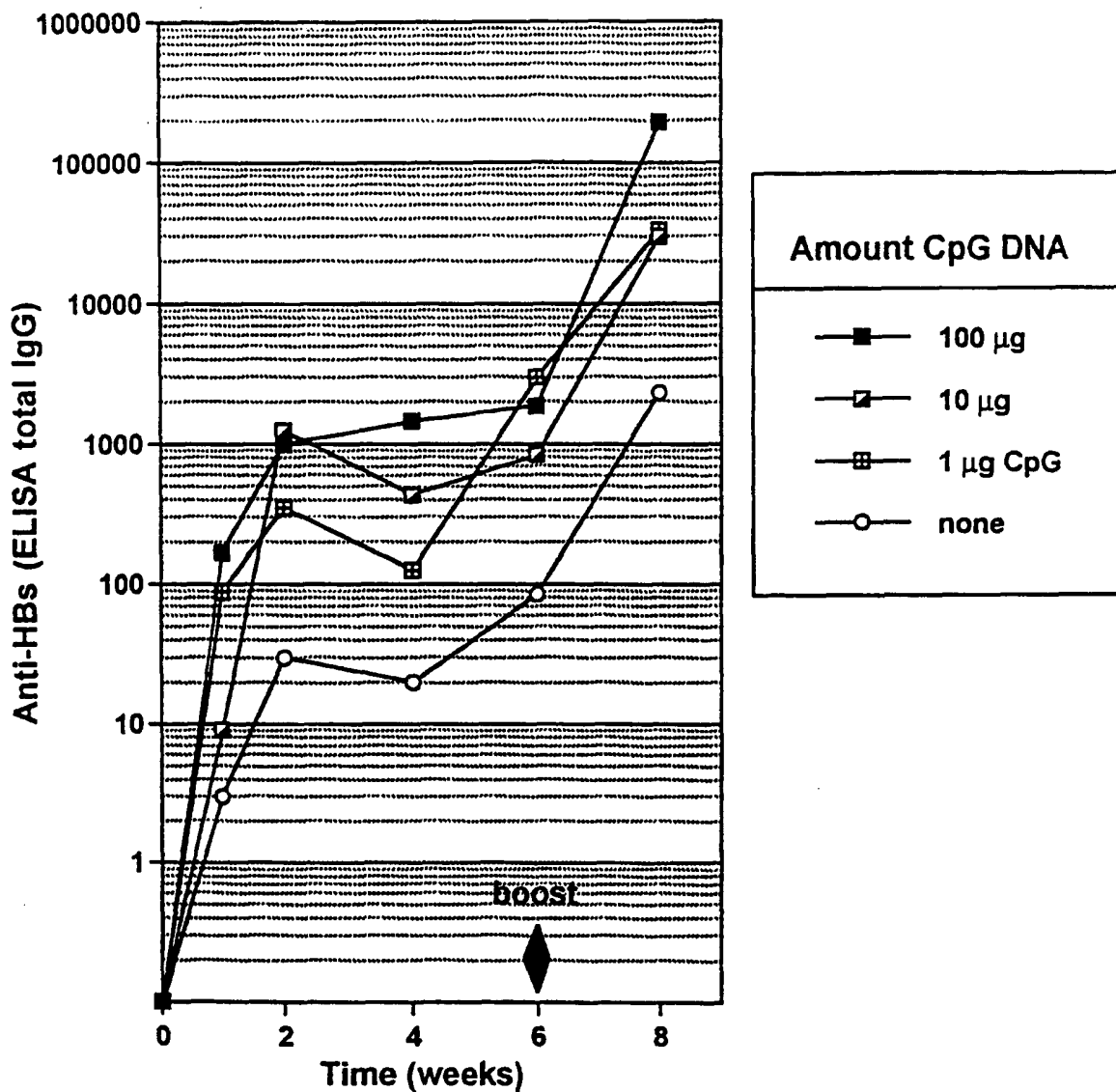
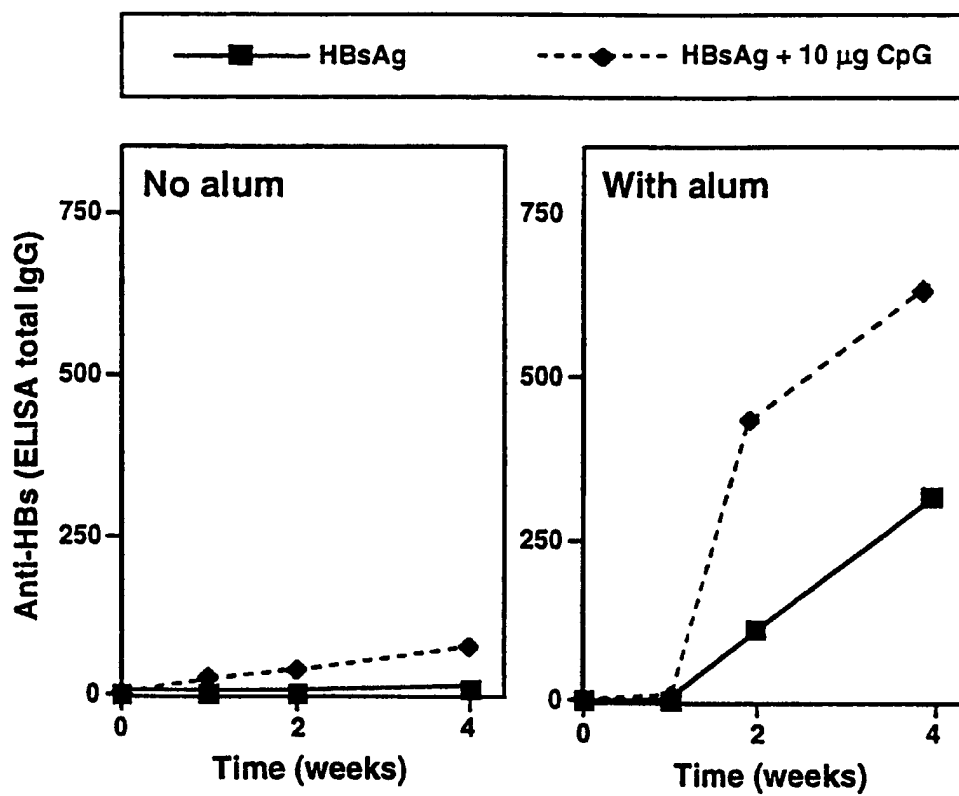


Figure 4

**Immunization of hypo-responder mice (B10.S)  
against HBsAg:  
Adjuvant effect of alum and/or CpG DNA**



**Figure 5**

# Immunization of non-responder mice (C2D) against HBsAg: Adjuvant effect of alum and CpG DNA

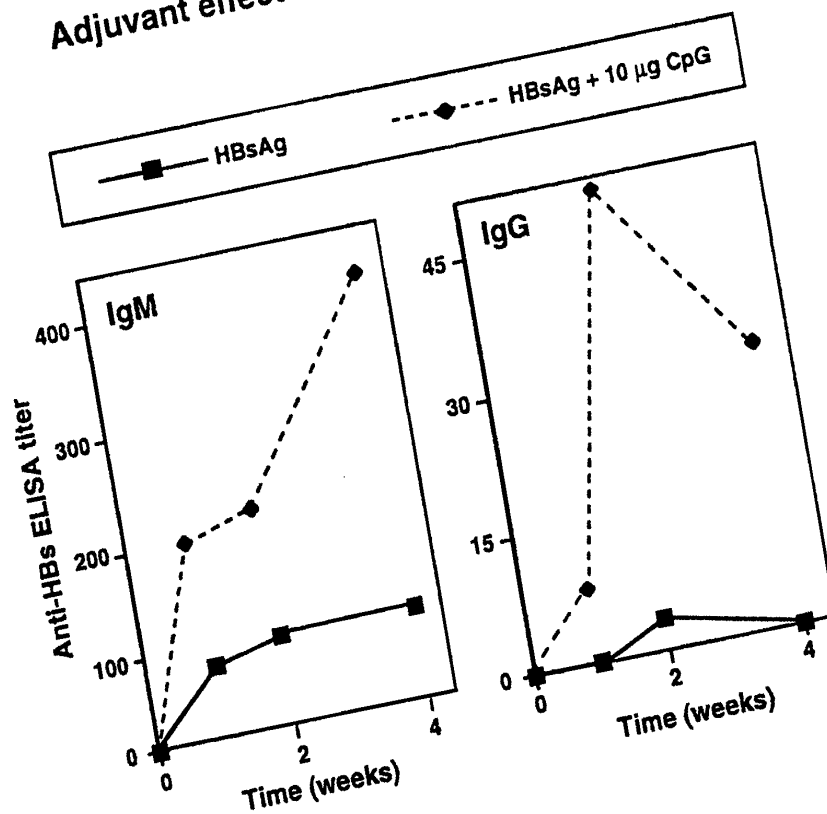
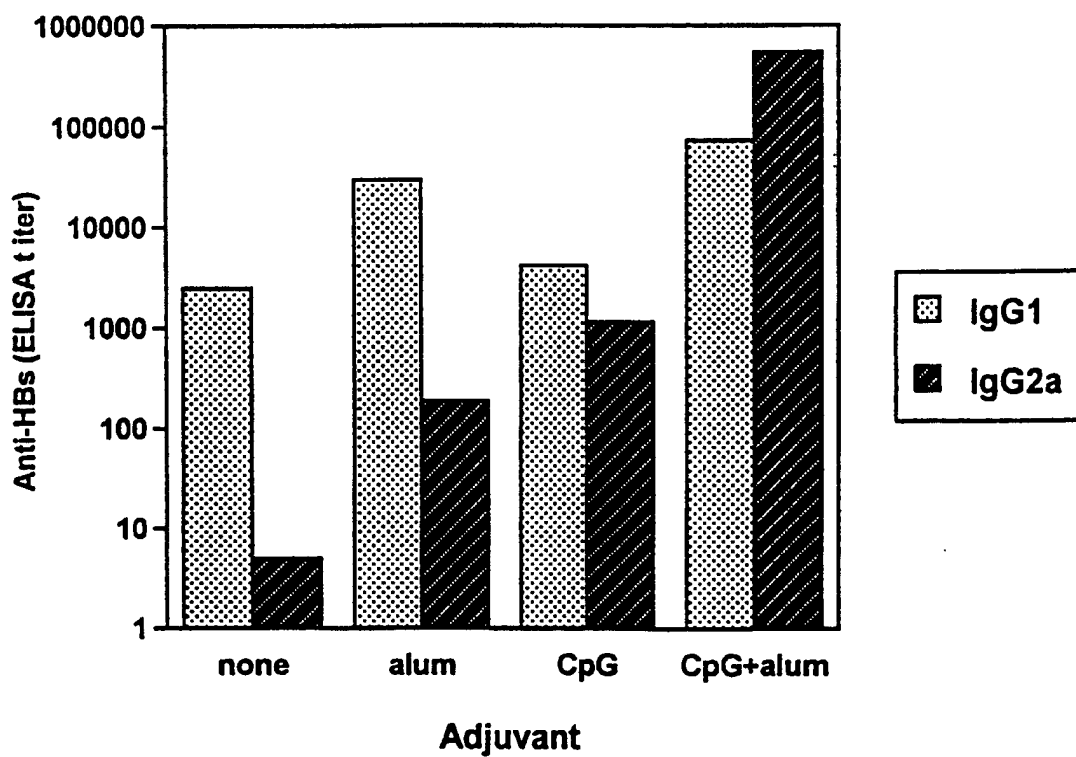


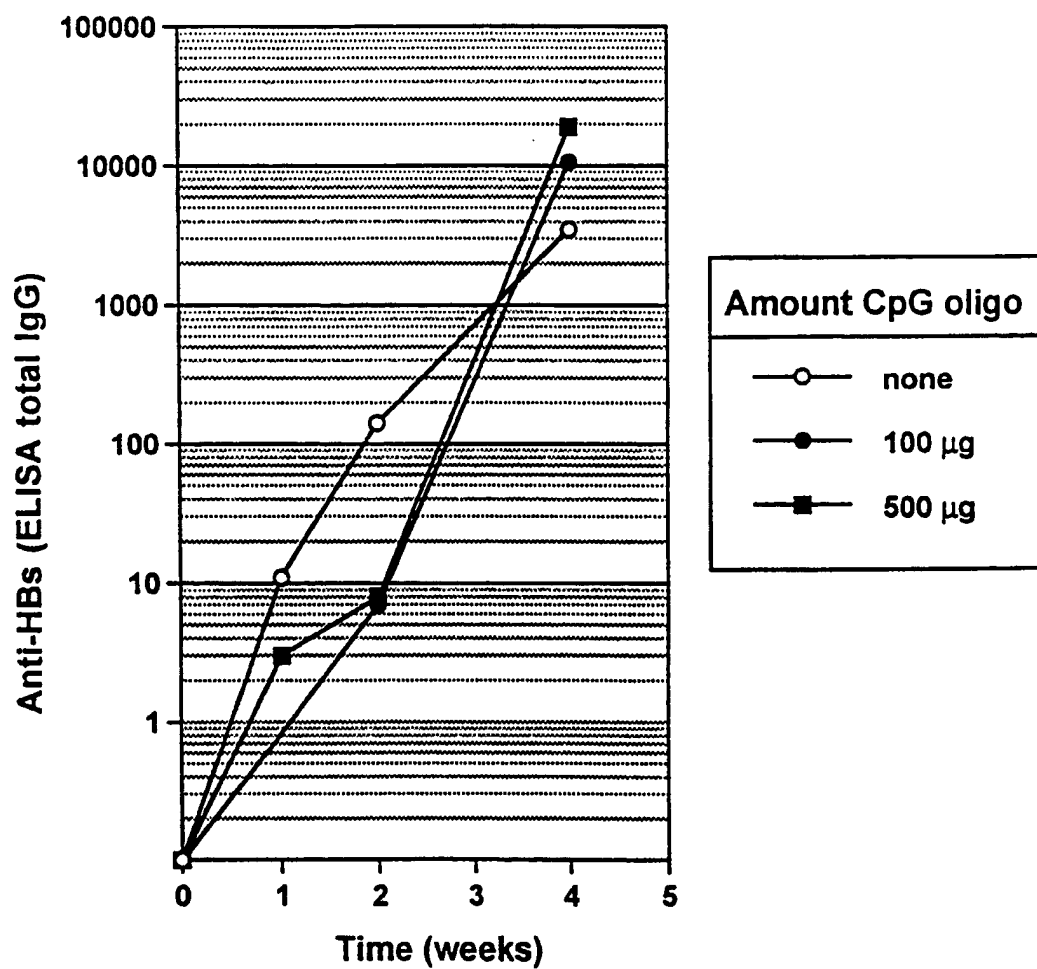
Figure 6

**Immunization of C57BL/6 mice against HBsAg  
Effect of adjuvant of antibody isotype**



**Figure 7**

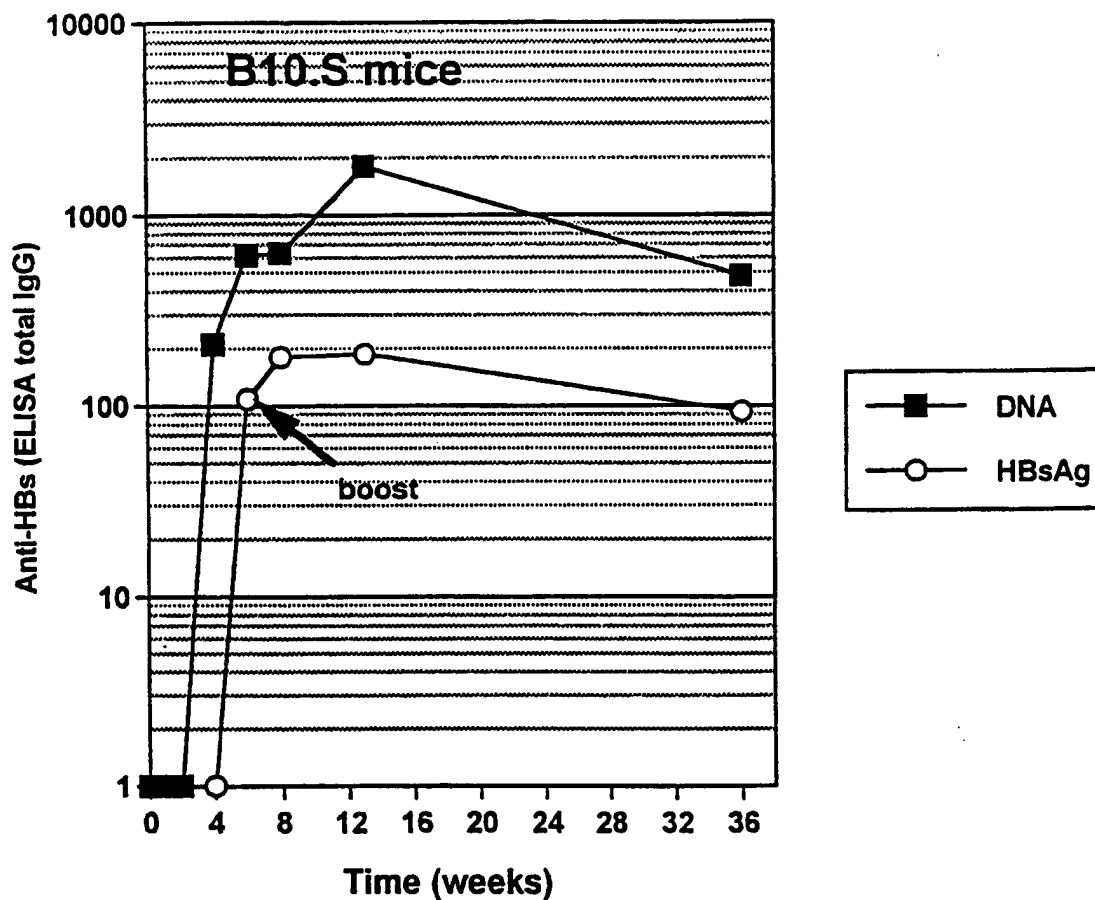
**DNA immunization of BALB/c mice against HBsAg  
Use of CpG oligos as adjuvant**



**Figure 8**



**DNA-based immunization of  
hypo-responder mice against HBsAg with  
protein (subunit) or DNA vaccines**



**Figure 9**

## Immunization of newborn BALB/c mice against HBsAg DNA vs protein vaccine

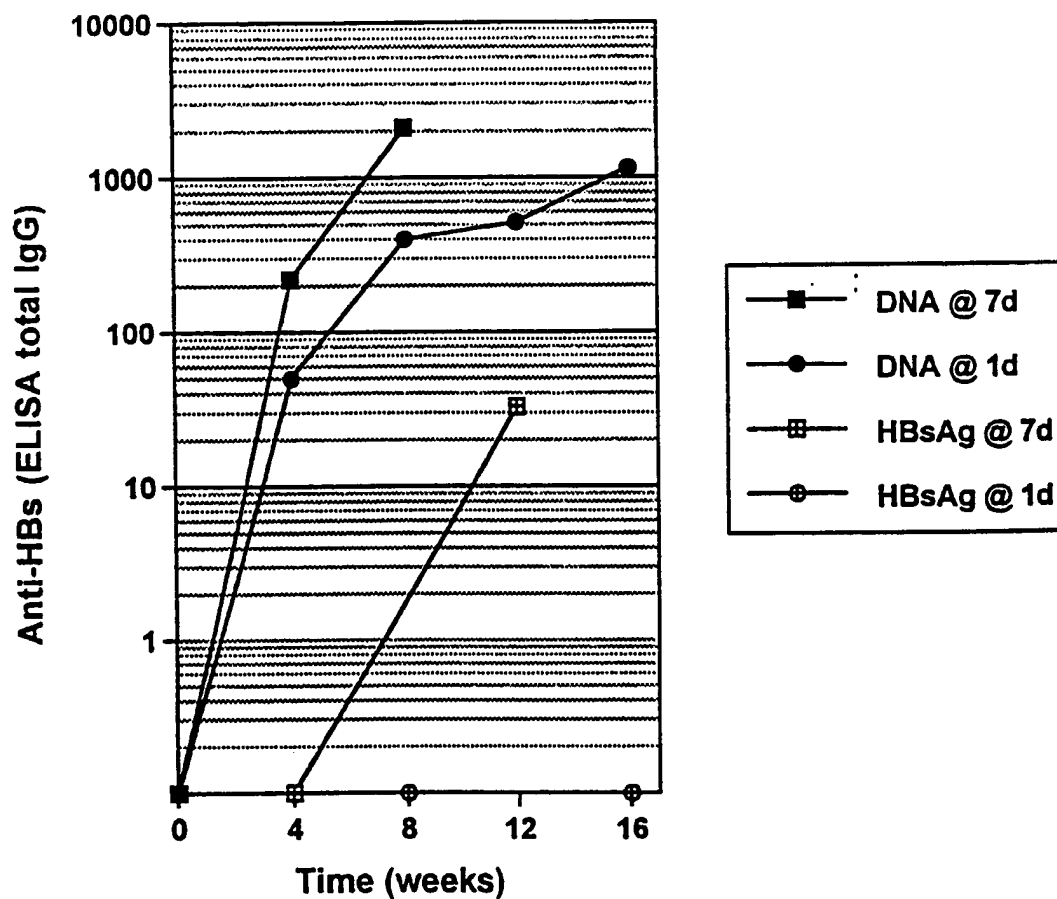
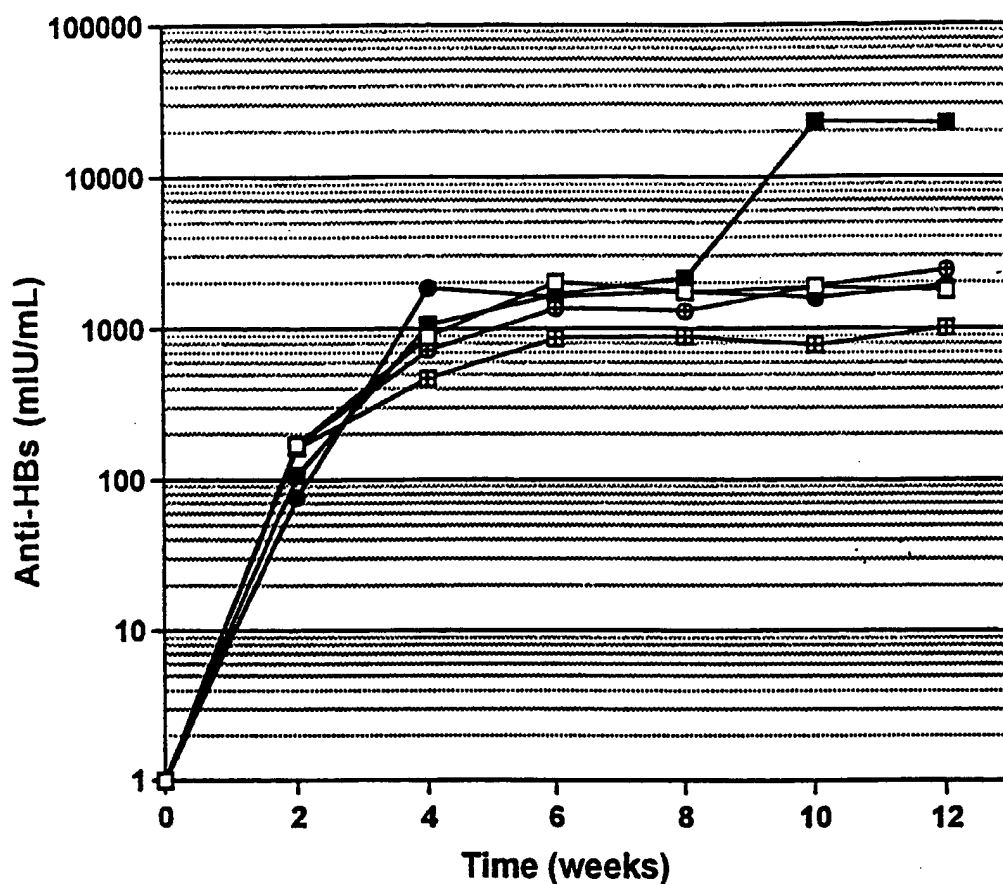


Figure 10

# Immunization of BALB/c mice against HBsAg by combined DNA and subunit vaccine approach



PRIME		BOOST
—□—	DNA	none
—●—	DNA + HBsAg (same site)	none
—■—	DNA, HBsAg (different sites)	none
—●—	DNA	HBsAg @ 2 wks
—■—	DNA	HBsAg @ 8 wks

Figure 11

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/04703

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 39/39

US CL :424/278.1, 184.1; 514/44; 536/23.1, 24.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/278.1, 184.1; 514/44; 536/23.1, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, Caplus, WPIDS, Scisearch, Europatfull

search terms: CpG, unmethylated, immune, adjuvant, hepatitis B

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BRANDA, R. F. et al. Amplification of Antibody Production by Phosphorothioate Oligodeoxynucleotides. Journal of Laboratory and Clinical Medicine, 1996. Vol 128, No.3 pages 329-338, see especially abstract and discussion page 336, second column.	1, 2, 4, 5, 8 --- 3, 6, 7, 9-45
X, P --- Y, P	WO 97/40163 A1 (SCHORR, J. et al.) 30 October 1997, pg. 12, third paragraph and examples.	1, 2, 4, 5, 8 --- 3, 6, 7, 9-45
Y, P	US 5,663,153 A1 (HUTCHERSON et al.) 02 September 1997, col 4 lines 33-52, and all examples.	1-45

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 JUNE 1998

Date of mailing of the international search report

16 JUL 1998

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